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(54) Title: NON-INVASIVE DELIVERY OF POLYPEPTIDES THROUGH THE BLOOD-BRAIN BARRIER, AND *IN VIVO* SELECTION OF ENDOCYTOTIC LIGANDS

(57) **Abstract:** A treatment method and genetic vectors are disclosed for non-invasive delivery of polypeptides through the blood brain barrier (BBB), to treat brain or spinal tissue. A genetic vector is used to transfect one or more neurons which "straddle" the BBB, such as sensory neurons, nocioceptive neurons, or lower motor neurons; this is done by administering the vector in a manner that causes it to contact neuronal projections that extend outside the BBB. Once inside a peripheral projection that belongs to a BBB-straddling neuron, the vectors (or some portion thereof) will be transported to the main cell body of the neuron, through a process called retrograde transport. Inside the main cell body, at least one gene carried by the genetic vector will be expressed, to form polypeptides. Some of these polypeptides (which can include leader sequences that will promote anterograde transport and secretion by BBB-straddling neurons) will be transported by the neurons to secretion sites inside the BBB. The polypeptides will be secreted by transfected neurons at locations inside the BBB, and will then contact and exert their effects upon secondary "target" neurons located entirely within the BBB. By using this system, polypeptides that stimulate nerve growth or activity can be used to treat neurodegenerative diseases, impaired limbs in stroke victims, etc., and polypeptides that suppress neuronal activity can be used to treat unwanted excessive neuronal activity, such as neuropathic pain. This approach also provides new methods for delivering endocrine and paracrine polypeptides into the CNS, thereby allowing improved medical and reproductive treatments in humans, and improved ability to modulate growth, maturation, reproduction, or other endocrine-related functions among livestock, endangered species, and other animals.

WO 03/091387 A2

NON-INVASIVE DELIVERY OF POLYPEPTIDES THROUGH THE BLOOD-BRAIN BARRIER, AND IN VIVO SELECTION OF ENDOCYTOTIC LIGANDS

FIELD OF INVENTION

This invention relates to methods for delivering polypeptides into brain and spinal tissue in humans and other mammals, and in other higher animals that have a blood-brain barrier (BBB). It also relates to methods for targeting delivery of polypeptides to specific populations or types of neurons entirely within the brain or spinal cord. It further relates to treatments for neurological disorders in humans, and to other forms of treatment (such as to regulate fertility, maturation, growth rates, etc.) in livestock and other animals, using polypeptides that normally cannot cross the BBB.

BACKGROUND OF THE INVENTION

The "blood brain barrier" (BBB) in higher animals helps ensure that neurons in the brain and spinal cord are not exposed to molecules in blood that would interfere with neuronal functioning if they penetrate the BBB. The BBB is not a single membrane. Instead, the capillaries in the brain and spinal cord are formed by endothelial cells that form "tight junctions". By contrast, capillary walls outside the brain and spinal cord have "slit-pores" between adjacent cells, which make those capillaries much more permeable. The BBB system is described in many textbooks and articles (e.g., Goldstein et al 1986; Pardridge 1998; Rubin et al 1999; Banks 1999; Kniesel et al 2000; Kniesel et al 2000). Animal models for studying BBB permeation are described in Bonate 1995, and early efforts to develop cell culture models are described in de Boer et al 1999.

Although the BBB protects the brain and spinal cord, it also excludes many therapeutic agents that could help treat diseases or injuries that affect the nervous system. In particular, with only a few exceptions, proteins and peptides cannot cross the BBB in any substantial quantities (e.g., Langer 1990). A small number of exceptions involve limited transport systems and certain types of neuronal receptors, such as transferrin receptors (e.g., Kastin et al 1999 and Granholm et al 1998). However, those limited exceptions are

not adequate for treating CNS disorders using various types of polypeptides. The need for better methods to transport therapeutic polypeptides (and other types of useful drugs) across the BBB is well-known, and many review articles have been published on the subject, include Zlokovic 1995, Cloughesy et al 1995, Davis et al 1995, Abbott et al 1996, Begley 1996, Kroll et al 1998, Pardridge et al 1998, Rochat et al 1999, and Rapoport 2000.

This invention relates to the delivery of polypeptides into brain or spinal tissue that is protected by the BBB. As used herein, *polypeptide* refers to any peptide molecule (formed by linking amino acids together) that has been formed, inside a living cell, by gene expression. Gene expression inside cells involves: (i) transcribing a DNA gene sequence to form a strand of messenger RNA (mRNA), followed by (ii) translating the mRNA strand to form a polypeptide chain made of amino acids bonded together in a precise sequence. Any such molecule formed by expression of a gene sequence is referred to herein as a polypeptide, regardless of whether the polypeptide is later processed by "post-translation" steps such as cleavage, cell secretion, glycosylation, cysteine crosslinking, etc.

Some scientists use "protein" to describe polypeptides that are complete and functional (as distinct from polypeptide fragments, or polypeptide precursors that have not yet been processed in ways necessary to render them active). In this invention, a polypeptide will not be of interest unless it is indeed capable of carrying out an intended function; therefore, the terms "polypeptide" and "protein" are used interchangeably herein.

A polypeptide is of interest herein (and is covered by the claims) only if it has three traits. First: as discussed below, the polypeptide must be expressed by a foreign gene (also called an "exogenous" gene) that has been inserted into one or more classes of neurons, using an intervention method such as described herein. However, a foreign (exogenous) gene carried by a vector may have exactly the same sequence as a "native" or "natural" gene that is expressed normally inside the CNS. This can occur, for example, in a treatment that increases levels of a polypeptide that is no longer being produced in adequate amounts, in a person suffering from a disease.

Second: the polypeptide must be expressed in one or more classes of neurons which are then capable of releasing the polypeptide inside brain or spinal tissue that normally is protected by the blood-brain barrier. As used herein, phrases such as "delivering polypeptides through a blood-brain barrier" or "releasing polypeptides inside (or into) brain or spinal tissue" indicate and require that the foreign polypeptide(s) must contact at least one cell or class of cells that reside wholly within the BBB. This requirement is not

satisfied if a polypeptide is secreted by a BBB-straddling neuron only at locations where the polypeptide contacts other BBB-straddling neurons. Instead, in most situations, this requirement will be satisfied only if a foreign polypeptide is secreted by a neuron into cerebrospinal fluid (CSF) and/or synaptic fluid, at a location inside the BBB.

Third: a foreign polypeptide is of interest if and only if it is therapeutic and/or otherwise useful, and has a desired and useful (rather than pathogenic) neuroactive property. This excludes viral infections or other non-useful infections, tests, and procedures that involve attacks or challenges to brain or spinal tissue. Many tests have been done on lab animals, using viruses that infect neurons or glial cells, to test antiviral drug candidates, and to perform "tracer" studies that analyze neuronal networks and connections by infecting animals with a virus such as rabies, then sacrificing the animals after a number of hours and analyzing samples of brain tissue, to see which neurons were infected by the viruses. Although such tests have used viruses to introduce foreign polypeptides into BBB-protected brain or spinal tissue, it should be clear that inflicting those types of infections on research animals (most of which were killed, as part of the experiment) were pathogenic, rather than therapeutic. Accordingly, this invention is substantially different from such tracer and other pathogen tests, since this invention is designed to provide a method for introducing therapeutic or otherwise useful and beneficial polypeptides into BBB-protected tissue, in fields such as human medical therapy, and in breeding of livestock or endangered species.

The reference to "neuroactive" is also a necessary part of the third factor discussed above. As used herein, "neuroactive" polypeptides are polypeptides that can exert a therapeutic or otherwise useful and/or desirable result or effect, if properly delivered to a desired and intended region of brain or spinal tissue that is protected by the BBB. Various examples are discussed below and listed in the tables herein.

It should be noted that useful neuroactive polypeptides may include polypeptides that will exert direct effects only on glial cells, without requiring direct effects on neurons, if the treated glial cells will generate a response that leads to a therapeutic or other beneficial result among neurons that are in fluid communication with the treated glial cells.

Also, the term "polypeptide" include variants, derivatives, and fragments of naturally occurring or genetically engineered polypeptides. Examples include: (i) chimeric or fusion polypeptides, which have a neuroactive portion derived from one gene, and a "leader" sequence (to increase stability, transport, secretion, or some other useful trait or activity) from some other gene; and, (ii) fragments or portions of polypeptides (such as a

single-chain binding fragment which has been isolated from the variable domain of a monoclonal antibody) that have a desirable form of neuroactivity.

The following section offers an overview of four categories of neuroactive polypeptides that can offer therapeutic benefits in people or animals suffering from neurological disorders.

Neurotrophic Factors

The root word "-troph" comes from the Greek work for "nourishment" or "food". "Trophic" implies that a certain molecule is involved in the stimulation, growth, nourishment, sustenance, or similar support of a certain system. "Neurotrophic factors" are molecules that promote neuronal growth, cause the formation of new synaptic connections between neurons, or carry out other stimulating or supporting neuronal activities. However, this term excludes: (i) nutrients (such as oxygen, glucose, amino acids, and nucleotides) that are required by all cells, and (ii) neurotransmitters which directly modulate nerve impulses between neurons (such as glutamate, acetylcholine, dopamine, serotonin, etc.).

Most neurotrophic factors function in a manner similar to hormones; they are secreted by one type of cell, and subsequently interact with other cells. However, they differ from hormones in that, after secretion, they typically interact only with neighboring cells. In some respects, they act in a manner comparable to "paracrine" factors; this term describes hormone-like molecules that act only locally, while "endocrine" factors act on the entire system and can affect cells distant from the secreting cell.

Neurotrophic factors are discussed in articles such as Lindsay 1994, Snider et al 1994, Bothwell et al 1995, Lewin et al 1996, and Skaper et al 1998, and in numerous US patents. The first neurotrophic factor that was discovered to stimulate nerve cell growth was called "nerve growth factor" (NGF). Later, other neurotrophic factors were discovered, and they required more elaborate names, such as brain-derived neurotrophic factor (BDNF), glial-cell-derived neurotrophic factor (GDNF), ciliary neurotrophic factor, and names with numbers in series, such as neurotrophin-3, neurotrophin-4, etc.

Many neurotrophic factors are polypeptides, and these offer potential therapies for many CNS disorders, including: (1) treatments for brain damage caused by physical injuries, such as automobile accidents, concussions, etc.; (2) brain damage caused by a stroke, cardiac arrest, near-drowning or suffocation, loss of blood, or other problems involving ischemia (inadequate blood flow) or hypoxia (inadequate oxygen supply); and, (3)

neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, etc. These potentials are discussed in articles such as Hefti 1994, Thoenen et al 1994, Ibanez et al 1995, McMahon and Priestley 1995, and Yuen and Mobley 1996.

For convenience, terms herein such as disorder, damage, and injury refer to any CNS disorder (whether from trauma, disease, etc.) that might be prevented or treated by one or more therapeutic polypeptides, if such polypeptides can be delivered to the proper regions of the brain or spinal cord.

Hypothalamic Releasing Factors

The hypothalamic releasing factors are short polypeptides that are made and secreted by nerve cells in the hypothalamus. These polypeptides in turn stimulate the pituitary gland to secrete various hormones that control and affect numerous important body functions involved in growth, metabolism, water and salt balance of the blood, reproduction, etc. are potent, and are the subject of considerable study. Articles that describe hypothalamic releasing factors include Turnbull et al 1999, Phelps et al 1999, and Sawchenko et al 2000.

Peptide Neurotransmitters

A number of peptide neurotransmitters have been described, including Substance P, enkephalins, endorphins, vasointestinal peptide (VIP), calcitonin gene related peptide (CGRP), galinin, somatostatin, etc. In many cases, peptide neurotransmitters act in a manner similar to classical neurotransmitters, such as glutamate or acetylcholine; they are released by a neuron at a synapse, where a binding reaction to a specific receptor on the other neuron at the synapse stimulates or inhibits the transmission of a nerve signal (also called a nerve impulse, firing, or depolarization). In other cases, binding of a peptide neurotransmitter to a receptor at a synapse exerts a more prolonged effect, such as increasing or decreasing the sensitivity of the target neuron to other neurotransmitters. In the perception of pain, for example, Substance P is released by nocioceptive (pain-transmitting) nerve fibers in the spinal cord, and is excitatory, while enkephalins and endorphins have the opposite effect, and inhibit transmission of pain signals. The pain relief provided by morphine arises from binding to endorphin receptors; however, the use of endorphins for the treatment of pain is not possible at present, because of the difficulty in safely delivering such peptides into the brain.

Cytokines and Other Growth Factors

Some cytokines and other growth factors can function as neurotrophic factors, by acting locally to stimulate nerve growth. However, cytokines can be distinguished from neurotrophic factors by their ability to stimulate growth and mitosis of various non-neuronal and dividing cells relating to the nervous system. Examples include acidic and basic fibroblast growth factor (aFGF and bFGF), epidermal growth factor (EGF), platelet derived growth factor (PDGF), insulin-like growth factor-I and II (IGF-I and IGF-II), tumor necrosis factor-B (TGF-B), leukemia inhibitory factor (LIF), various interleukins, etc.

These molecules are involved in normal physiological processes such as CNS tissue growth, remodelling and repair after injury, and immune responses within the CNS. While they have potential to be used to treat CNS injuries or infections, they pose a risk of stimulating cancer cells, especially if administered in a systemic fashion throughout the CNS. Nevertheless, they are of great interest to neurology researchers, and development of methods for delivering cytokines and similar molecules to discrete and limited CNS regions would be of great interest and potential benefit.

Table 1 provides a partial listing of known CNS-active polypeptides. To illustrate one area of application in slightly more detail, Table 2 provides examples of some CNS disorders, along with the polypeptides that are currently thought to have great promise in treating or preventing these disorders. Tables 1 and 2 contain only partial listings, and numerous research efforts (including the human, mouse, and rat genome sequencing projects) are rapidly leading to the identification of numerous other neuroactive polypeptides.

The biggest and most difficult obstacle that is hindering the evaluation of neuroactive polypeptides, and the use of such therapies in patients who need such help, is the severe difficulty of actually delivering neuroactive polypeptides to specific regions of the brain and spinal cord where those polypeptides must be able to contact specific and targeted populations of neurons or glial cells in order to provide a therapeutic, preventative, or other benefit. Accordingly, the purposes and goals of this invention are: (i) to provide methods for delivering foreign polypeptides into areas of brain and spinal tissue that are protected by the BBB, and (ii) to provide methods for delivering foreign polypeptides to targeted, specific, and limited regions of brain and spinal tissue, and/or to targeted, specific, and limited types or classes of cells inside the CNS.

PRIOR ART CNS DRUG DELIVERY METHODS

Reviews of the drug delivery literature (e.g., Langer et al 1989; Langer 1990; Madrid et al 1991; Thoenen et al 1994; Zlokovic 1995, Cloughesy et al 1995, Davis et al 1995, Abbott et al 1996, Begley 1996, Kroll et al 1998, Pardridge et al 1998, Rochat et al 1999, and Rapoport 2000) identify few currently available, effective, and clinically practical alternatives for delivering polypeptides across the blood brain barrier. Various approaches to delivering drugs into the BBB that have been tried, without great success, are reviewed in articles such as Zlokovic 1995 and Rapoport 2000. These efforts can be categorized and summarized as follows:

1. *Invasive methods*, which require physical intrusion, such as surgery, or at a minimum, use of a hypodermic needle to puncture cells and membranes. One example is intraventricular injection or infusion, which require a neurosurgeon to gain access to one of the "ventricles" that serve as production sites or gathering nodes for cerebrospinal fluid, so that a drug can be injected into the fluid that has accumulated in a ventricle. Most of these ventricles are buried deep inside the brain (the fourth ventricle is somewhat accessible in humans, but it is at an exit point for fluid leaving the brain, so it is not useful for delivering drugs to most of the brain). Injecting drugs into brain ventricles, even if only with a needle, is difficult and dangerous. Any penetration by a blade or needle will damage capillaries along the way, and if blood leaks out from damaged capillaries, it will contact neurons that must be protected from blood by the BBB. If cautery is used to minimize blood leakage, capillaries and small arteries and veins that are cauterized will no longer carry blood, and neurons served by those blood vessels may be killed.

Another invasive method involves transplantation of genetically selected and/or engineered cells, which can secrete neurotrophic molecules at high rates, into target regions of the brain (e.g., US Patent 5,082,670, Hefti et al 1992). The insertion of such cells into a target portion of the brain requires invasive access, using blades or needles, and poses serious problems and dangers. Because of these problems, these invasive techniques are cannot be used in humans except in very limited clinical trials, in patients suffering from terminal diseases, or diseases that are so severe and debilitating that a patient cannot be significantly helped by any other treatment.

2. *Non-invasive methods*, which use chemical, cellular, carrier-mediated, or other non-surgical methods to transport neurotrophic or similar molecules across the blood-brain barrier. These methods typically require fairly large quantities of vesicles, complexes, or

other macromolecules to be injected into the bloodstream, in the hope that some quantity of the injected "passenger" molecules will enter the brain and be transported across the BBB, into brain tissue. Examples of these approaches are described in items such as US patent 5,154,924 (Friden 1992), a chapter by Thoenen et al in Racagni et al 1994, and Zlokovic 1995.

One of the main problems with these approaches is that if a certain compound can pass through the BBB in a significant quantity, that same compound will also pass through the much more permeable capillaries in the rest of the body, faster and in larger quantities. This leads to two serious problems. The first involves side effects that occur when large quantities of neuroactive compounds are delivered into non-CNS tissue; such compounds can disrupt the peripheral nervous system, or other types of cells or tissue. The second set of problems relate to the very high expenses that arise when large quantities of complex drugs must be carefully synthesized, purified, and quality-tested before injection into a human. In addition, such injections or infusions usually must be repeated, often numerous times, and after an injection or infusion of a large quantity of a neuroactive compound, a patient must be monitored for at least a day or more, usually in a hospital or clinic setting rather than at home, so that emergency measures may be taken immediately if the patient suffers an adverse reaction.

Also, the prior methods do not allow foreign polypeptides to be administered only to selected neurons, classes of neurons, or brain regions. By contrast, this invention offers a number of promising approaches to administering foreign polypeptides to targeted neurons in a controllable and selective (or at least enriched) manner. By targeting only certain locations or regions inside the CNS, and/or by targeting only certain types or clusters of neurons, the risk and severity of side effects can be minimized.

GENE THERAPY

One approach that has potential for treating some CNS disorders involves gene therapy. In prior efforts to genetically modify brain and spinal neurons, two main approaches have been used. In one approach, cells are genetically altered, outside the body, and then transplanted somewhere in the CNS, usually in an area inside the BBB. In the other approach, genetic "vectors" are injected into one or more regions in the CNS, to genetically alter cells that are normally protected by the BBB. Because serious obstacles arise when attempts are made to adapt procedures that work well, in other parts of the

body, to the special challenges of treating brain or spinal tissue, these two types of approaches are described in more detail below.

Transplant of Genetically Engineered Cells

In conventional tissue, cell transplants usually involve isolating a population of cells, manipulating those cells under cell culture (*in vitro*) conditions, and then transplanting the manipulated cells into the animal or patient. In some cases, the cells are obtained from the host, to minimize potential rejection problems after the cells are implanted back into the host. In other cases, they may be from other sources (such as an immortal cell line, fetal or stem cells, etc.). The genetic manipulation may involve precise and controlled techniques, or it may involve "shotgun" approaches followed by screening tests.

Using this general approach in CNS tissue is generally not practical, for a number of reasons, including the following brief listing. First: the CNS is heterogeneous in the extreme, and nervous tissue from one region can not be substituted for nervous tissue from another region. Second: CNS neurons in adults are post-mitotic, and will not divide and repopulate if a vacancy is created. Third: nerve cell processes or axons, once broken, do not readily regrow and reestablish synaptic and other connections. Fourth: surgical or similar extraction of CNS tissue poses great risks to a patient, even if the surgery is done with the highest level of skill and care.

For these and other reasons, in the vast majority of cases, it simply is not feasible or practical to remove a population of CNS neurons from a patient, genetically manipulate the neurons, and then return them back into the patients's brain or spinal cord. Therefore, most experiments to implant genetically engineered cells into CNS tissue have not even attempted to use cells taken from the same patient. Instead, early tests used genetically engineered cells (not necessarily neurons) that had been modified to secrete abnormally large quantities of some desired protein (such as nerve growth factor, for transplantation into the brains of patients suffering from Alzheimer's disease). These types of tests and treatment efforts are described in articles such as Blesch and Tuszynski 1996, Karpati et al 1996, Fick and Israel 1994, Fisher and Ray 1994, and Friedman 1994, and in patents such as US 5,082,670 (Hefti et al 1992). Although this research is interesting and may become useful some day, major risks and dangers will be unavoidable, whenever foreign cells must be implanted inside CNS tissue. Any such implants must puncture or cut through brain or spinal tissue, and the process of implantation will necessarily injure the brain or spinal tissue that must be

penetrated or displaced.

By contrast, genetic manipulation of CNS cells, without moving the cells (called *in situ* treatment) offers better hope for correcting a problem without inflicting invasive damage on brain or spinal tissue.

Genetic Alteration of CNS Cells *In Situ*

This approach aims to introduce into the CNS a source of a desirable polypeptide, by genetically altering cells within the CNS without moving those cells. In the prior art, this has been achieved, a number of times, by directly injecting genetic vectors carrying foreign genes into brain or spinal tissue. However, this approach is invasive; brain or spinal tissue must be punctured or cut, somehow, by a needle or blade. What is needed, instead, is a non-invasive method of genetically transfecting or transforming cells that sit inside the BBB, without having to puncture, cut, or otherwise invade or penetrate any tissue that is protected by the BBB.

The terms "transfect" and "transform" are used interchangeably herein. Both terms refer to a process which introduces a foreign ("exogenous") gene into one or more preexisting cells, in a manner that causes the foreign gene(s) to be expressed to form polypeptides. To some scientists, "transfect" implies that any foreign gene(s) will be expressed only transiently, in a manner analogous to an infection that lasts only for a while and eventually is stopped. By contrast, "transform" implies a permanent genetic alteration that will be passed on to any and all progeny cells, usually due to integration of the foreign gene(s) into one or more cell chromosomes. However, the boundary lines between those terms are blurred, and the distinctions between those terms are not used consistently by all scientists. Accordingly, "transfect" and "transform" are used interchangeably herein, regardless of how long a foreign gene might continue to be expressed after it enters target cell(s).

GENE VECTORS

There are two broad classes of genetic vectors. The viral vectors are derived from viruses, and make use of the lipid envelope or surface shell (also known as the capsid) of a virus. These vectors use a virus's ability to bind to one or more particular surface proteins on certain types of cells, and then inject the virus's DNA or RNA into the cell. These have become the dominant class of vectors that have been used in attempts at human gene

therapy, and they are described in numerous published articles. Gene transfers into CNS neurons have been reported, using vectors derived from herpes simplex viruses (e.g., European Patent 453242, Breakfield et al 1996), adenoviruses (La Salle et al 1993), and adeno-associated viruses (Kaplitt et al 1997).

All other genetic vectors are generally grouped into a broad class of non-viral vectors. These vectors typically contain a "gene expression construct", which is understood by those skilled in the art. By way of illustration, such a construct might be a plasmid, which normally would contain (i) a bacterial origin of replication, so it can be grown quickly in host cells such as *E. coli*; (ii) at least one selectable marker gene, to allow host cells containing the plasmid to inactivate an antibiotic, grow on lactose without requiring any other carbon source, or turn color when certain chemicals are present, and, (iii) at least one passenger gene, containing a suitable promoter and a coding sequence that will express the desired polypeptide, after the gene expression construct has been inserted into targeted mammalian cells.

A non-viral genetic vector is created by adding, to a gene expression construct, agents that will enable and promote entry of the gene construct into target cells. Several commonly used agents include cationic lipids, positively charged molecules such as polylysine or polyethylenimine, and/or ligands that bind to receptors expressed on the surface of the target cell (such as, in some cases, a viral ligand that was originally obtained from a virus). Major categories of non-viral gene vectors include:

1. cationic gene vectors. DNA strands and cell surfaces are both negatively charged. Therefore, positively-charged agents can help DNA strands overcome that electrical repulsion, and enter target cells. Examples include polylysine, polyethylenimine (PEI), and various cationic lipids. These agents are described in articles such as Sahenk et al 1993, Nabel et al 1997, and Li et al 2000.

2. receptor-targeting gene vectors, in which a strand of DNA is bonded to a molecule that normally has its own way to enter certain types of cells, through receptor molecules on the surfaces of certain cells. This approach was demonstrated in liver cells in US patent 5,166,320 (Wu et al 1992). By using this approach, DNA delivery can be targeted to a particular type of cell, by coupling DNA to a molecule that is selectively taken up by that type of target cell. This process depends on cell receptors, which typically are proteins that straddle the outer membrane of a cell, with part of the receptor protein exposed outside the cell, and part of the protein anchored inside the cell cytoplasm. To be

suitable for this type of uptake, the receptor must undergo a process called "endocytosis", which means that, when a suitable ligand binds to the receptor, a process is triggered that cause both the receptor, and the ligand that is bound to it, to be pulled into the cell.

Since endocytosis is of substantial importance in one aspect of this invention (which involves a new type of *in vivo* screening method that uses nerve fibers to identify ligands that can activate and drive endocytosis), it is discussed in more detail below.

3. Other non-viral genetic vectors. Since viral vectors suffer from problems and limitations, and since cationic compounds and receptor-targeting ligand vectors currently are not nearly as efficient as viral vectors in leading to expression of foreign genes by target cells, researchers have attempted to develop ways to enhance gene delivery into cells. One such effort involves the use of viral capsid proteins that can rupture the "endocytotic vesicle" that is formed when a foreign molecule or particle is drawn through a cell's outer membrane, through endocytosis. Before foreign DNA can begin acting as a gene, it must escape from the endocytotic vesicle, and some types of viruses (including adenoviruses) have evolved capsid molecules that rupture endocytotic vesicles, thereby releasing the foreign DNA inside the cell cytoplasm. An example of this type of effort is described in Curiel 1997.

Various other efforts are being made in other areas, but none of those efforts have yet managed to reach the mainstream of research on genetic vectors.

VIRAL CNS INFECTIONS AND TRACER STUDIES

The fact that some viruses (such as rabies and polio viruses) can spread from neuron to neuron, in CNS tissue, has allowed researchers to use viral spreading to track neuronal systems and pathways, using "trans-neuronal tracing"

There are important similarities between the patterns of motor neuron infections by polioviruses, and the patterns of motor neuron degeneration in patients suffering from amyotrophic lateral sclerosis. Similarly, the degeneration of basal forebrain cholinergic neurons that is an early feature of Alzheimer's disease shows important similarities to damage to certain neurons after administration of rabies virus to the olfactory epithelium in the nose. These previously unpublished observations are disclosed herein, because the similarities between patterns of viral infection in CNS tissue, and patterns of neurodegeneration in certain CNS disorders, suggests that the methods disclosed herein (i.e., non-invasive delivering of therapeutic polypeptides into the brain or spinal cord, in a

manner which relies on certain neurons to transport and deliver polypeptides to other neurons that share contact points with the carrier neurons) offers the promise of both: (i) powerful therapeutic treatments, and (ii) powerful experimental approaches that can be used by researchers to, for example, selectively alter patterns of gene expression and/or nerve cell function within brain or spinal tissue, and/or study various networks and principles within CNS tissue, including factors that govern the response of CNS tissue to assaults ranging from viral infections to neurodegenerative diseases.

ENDOCYTOTIC SURFACE MOLECULES

One aspect of this invention involves the discovery and disclosure of a new method for *in vivo* screening and identification of "ligand" molecules that can be used to activate and drive the process of endocytosis (i.e., transport of a foreign molecule, into a cell interior) These types of ligand molecules can be regarded as vehicles, carriers, or transport systems, that can be used to pull or carry useful "passenger" or "payload" molecules into specific targeted types of cells. These passenger molecules can be segments of DNA carrying foreign genes, if the goal is gene therapy or similar genetic manipulation to deliver polypeptides into brain or spinal tissue, or other targeted cells. However, this type of endocytotic transport is not limited to gene therapy, and also can be used to deliver therapeutic drugs, diagnostic compounds, or other useful agents into specifically targeted classes of cells.

This type of endocytotic transport (also referred to interchangeably as internalisation) mainly involves a class of proteins known as "receptor" proteins, which are present on the surfaces of cells. However, at least some types of other surface molecules are known to also trigger the process of endocytosis (as examples, tetanus and cholera toxins are known to bind specifically to particular surface carbohydrate molecules, in a manner that activates and drives internalisation of the resulting ligand-carbohydrate complex). Therefore, although most references herein are to endocytotic receptor proteins as the main exemplary class that will be used to explain and illustrate the invention, it should be kept in mind that certain other types of endocytotic surface molecules can also be used as the targets of endocytotic ligands as discussed herein.

The structures of cell membranes and endocytotic receptor proteins, and the process and mechanisms of endocytosis, are discussed in various reference works, such as Alberts et al, *Molecular Biology of the Cell*. In Alberts et al's third edition, 1994, the relevant

pages include 478-488, 618-625, and 731-744 (which discuss cell membranes and receptor proteins), and pages 618-626 and 636-641 (which describe the proteins and processes involved in endocytosis). It should also be noted that endocytosis can include pinocytosis (if the ligand which is being taken inside the cell is relatively small) or phagocytosis (if a larger item, such as an entire cell or viral particle, is being taken into a cell).

Not all candidate ligands that bind to an endocytotic receptor protein can drive and complete the process of endocytosis. As an example, an important class of "low affinity" nerve growth factor receptors, known as p75 receptors, has been identified in mammals, and monoclonal antibodies that can bind to these receptors have been created by numerous research teams. However, most of those monoclonal antibody lines cannot drive and complete the process of endocytosis, after they bind to p75 receptors. Only a few particular monoclonal antibody preparations are internalised by p75 receptors; this includes a particular line known as MC192 antibodies, initially disclosed in Chandler and Shooter 1984, and later reported to be internalised by p75 receptor bearing cells in Yan et al 1988.

Substantial work has been done to identify ligands that can enable endocytotic transport into mammalian cells. Much of that work involves a class of genetic research tools called phages, and phage display libraries. Very briefly, phages (this word is short for bacteriophages) are viruses that can infect and reproduce in bacteria. Certain types of phages which have long shapes, known as "filamentous phages" (abbreviated as "Ff" phages) were studied and developed by genetic researchers, because they can be engineering to express foreign proteins, that will be accessible (displayed) on the surfaces of phage particles that remain viable and infective.

One strain of these filamentous phages was manipulated to give it a bacterial plasmid origin of replication, thereby converting it into a "phagemid". Its DNA can be reproduced in large quantities in *E. coli* cells, either in double-stranded form, as circular plasmids, or in single-stranded form, which can be packaged in phage coat proteins for secretion as new and infective phage particles without killing the host cells. This phage line was also given a kanamycin resistance gene, as a selectable marker, allowing *E. coli* cells that carry the phage to grow in culture media that contains kanamycin, an antibiotic that kills non-resistant cells. This phage line, designated as the M13 line, is commercially available, and is widely used in genetic research.

Various researchers and companies have created entire "phage display libraries", in which a large population of phages (such as M13 phages) is manipulated so that it will

display a wide variety of different foreign proteins. Two such display libraries were used by the Applicants herein, in their work to create and test an *in vivo* screening process to identify endocytotic ligands that could bind to targeted endocytotic receptors on the surfaces of neuronal fibers that extend outside the blood brain barrier. One of those phage display libraries, known as the scFv library, which contains roughly 13 billion different phagemids, each of which carries a foreign polypeptide sequence that normally appears in the antigen-binding "variable fragment" domains of human antibodies. The other phage display library known as the PhD-C7C library, carries small foreign polypeptide sequences containing 7 amino acids each, sequenced together randomly using a process called "combinatorial chemistry". Both of these two phage display libraries are described in more detail below, in the Detailed Description section, and the Examples.

Phage display libraries offer powerful and useful tools for screening huge numbers of different "ligand candidates" or other polypeptide sequences. They can help researchers identify, isolate, and then reproduce those few phages, in a huge library, that happen to carry a foreign polypeptide sequence that is of interest, because of some particular binding or cellular activity.

However, because of a number of cellular and physiological factors, it was very difficult or totally impossible (under the prior art) to do any screening of phage display libraries *in vivo* (i.e., in the bodies of living animals). Instead, nearly all such screening was limited to affinity columns (which contain immobilized antigens or antibodies, or similar binding reagents), or *in vitro* conditions (using cells in culture media).

As a result, when it came to efforts to identify and isolate ligand molecules that could activate and drive the process of endocytosis, the only real and substantial progress that had been created prior to this invention was in identifying ligands that could enable transport into cancer cells, and cells involving autoimmune diseases, because those two classes of cells can be grown and tested readily, in *in vitro* cell culture. Examples of such works include US patents 5,977,322 (Marks et al 1999), 6,113,898 (Anderson et al 2000), and 6,376,170 (Burton et al 2002), and PCT applications WO 2000-29004 (Plaksin), 2000-38515 (Ferrone), and 2000-39580 (Christopherson et al). There was no comparable progress in successfully screening phage display libraries to identify ligands that can drive endocytotic transport into other types of tissue cells, such as neurons.

The Applicants herein were searching for ways to genetically transform neurons (and, in particular, neurons that straddle the blood-brain barrier and have fibers that extend

outside the BBB). In addition, because they were aware of the problems and limitations that have plagued and thwarted the medical use of viral vectors, they were looking for other types of transport and delivery systems, which could pull strands of foreign DNA into cells (and, in particular, into neurons that straddle the BBB, and have fibers that extend outside the BBB).

All of these efforts came together, and have led to the invention disclosed herein.

Prior to this invention, despite the huge advances in molecular biology and neuroscience in recent years, there remains a major and unmet need for better methods of transporting useful polypeptides across the blood-brain barrier, so that they can provide therapeutic benefits to neurons in brains and spinal cords, without requiring invasive damage.

Accordingly, one object of this invention is to disclose an improved and non-invasive method for delivering polypeptides to targeted cells inside the BBB.

Another object of this invention is to disclose a new method of transfecting sensory neurons or motor neurons that "straddle" the BBB, in a manner which causes the BBB-straddling neurons to deliver therapeutic polypeptides to neurons which are located entirely within the blood-brain barrier.

Another object of this invention is to use genetic engineering methods and compounds to provide a method of transporting polypeptides into CNS tissue, in a manner that can be used to selectively affect targeted clusters, regions, or types of neurons located entirely within the blood-brain barrier.

Another object of this invention is to disclose new and practical *in vivo* methods for identifying and isolating ligand molecules that can be used to effectively transport "passenger" or "payload" molecules (including, but not limited to, DNA strands that encode foreign proteins) into selected, targeted, and limited types and classes of neuronal fibers, and other selected and targeted classes of animal cells.

Another object of this invention is to disclose a new method for identifying and isolating ligand molecules that can be used to effectively transport therapeutic drugs, diagnostic or analytical compounds, or DNA sequences, into selected, targeted, and limited types and classes of animal cells.

Another object of this invention is to disclose a new method for *in vivo* screening of libraries, repertoires, or other assortments containing multiple candidate polypeptides or other compounds that have been created by combinatorial chemistry, to identify and isolate

those particular candidates that undergo endocytotic transport into cells.

These and other objects of the invention will become more apparent through the following summary, drawings, and description of the preferred embodiments.

SUMMARY OF THE INVENTION

A method and compounds are disclosed for the noninvasive transport and delivery of polypeptides through the blood brain barrier (BBB) and into the central nervous system (CNS) of a mammal or other higher animal, in a manner which allows foreign polypeptides to contact cells located wholly within the BBB. This method enables previously unavailable forms of medical or veterinary treatment in humans and other animals, and it can also be used for commercial purposes, such as in livestock.

This method is accomplished by using a genetic vector to transfect a selected type of neuron which "straddles" the blood brain barrier. Examples of such BBB-straddling neurons include sensory neurons (such as olfactory receptor neurons, and nocioceptive neurons), and motor neurons; pre-ganglionic neurons of the autonomic nervous system may also be useful, in some treatments. Copies of the genetic vector are introduced and administered to the patient or animal in a manner that causes the vectors to contact and transfect neuronal "projections" that extend outside the BBB. After the vector enters a peripheral projection of a BBB-straddling neuron, a vector-borne gene encoding a CNS-active polypeptide will be transported by retrograde transport to the main cell body, where it will be expressed by the transfected neuron, to form therapeutic or otherwise useful polypeptide molecules. These polypeptides will be of a type that normally are secreted by cells, or they can be provided with leader sequences that can promote secretion; accordingly, they will be transported within the transfected neuron to one or more secretion sites located within CNS tissue that is protected by the BBB. The polypeptides that are secreted at such locations inside the BBB will then be able to contact targeted neurons that are located wholly within (and are therefore protected by) the blood-brain barrier.

Using this system, polypeptides such as neurotrophic factors can be noninvasively delivered to targeted types and classes of neurons that lie wholly within the BBB. This allows new methods of treating brain and spinal neurons that have been injured, or that are degenerating due to aging, disease, or other disorders. Alternately, this new method allows polypeptides that can suppress neuronal activity to be transported across the BBB, to reduce problems involving unwanted and excessive neuronal activity, such as neuropathic pain.

This approach also allows new methods of delivering polypeptides that can modulate endocrine functions into the CNS, thereby allowing improved treatment of various medical problems in humans, and improved ability to modulate growth, maturation, reproduction, or other endocrine-related functions among animals, including livestock and endangered species.

In addition, a new *in vivo* screening method is disclosed, for identifying and isolating endocytotic ligands that will be taken into, and transported within, neuronal fibers. This ligands can be used to prepare gene vectors that can deliver cells into selected BBB-straddling vectors. Briefly, this screening method uses the following steps: (1) emplacing multiple candidate ligands at a first location inside the body of a living animal, where the candidate ligands will directly contact nerve fibers (such as a sciatic nerve bundle, in the leg of a rat); (2) allowing enough time to pass for the nerve fibers to internalise those particular ligands which can activate and drive endocytosis; (3) harvesting segments of the nerve fibers, at a site (such as distal to a ligature that constricts the sciatic nerve, in a rat hip) that is sufficiently distant from the ligand emplacement site to avoid collecting ligand candidates that did not enter or were not transported by the nerves; and, (4) removing the internalised ligands from the harvested nerve segments.

This method can be carried out using phage display libraries (for polypeptide ligands), or other ligand repertoires that have been created by combinatorial chemical synthesis. Since it offers a preferred mode of carrying out this invention, it is disclosed and claimed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 depicts the use of a genetic vector to transfect an exposed projection ("mucosal tip") of an olfactory receptor neuron, for the purpose of delivering therapeutic polypeptides through the BBB to neurons that reside wholly within the BBB. The transfected olfactory receptor neuron will express the vector-borne gene into polypeptides, which will then be transported through a neuronal axon which crosses the BBB. The polypeptides will then be secreted inside the BBB, by the BBB-straddling neuron, where they can contact CNS neurons that are located wholly inside the BBB, such as cholinergic neurons in the basal forebrain.

FIGURE 2 depicts a viral vector which can transfect neurons; this vector includes a capsid shell, binding ligands on the surface, and a genetically-engineered genome which

contains a useful "passenger" or "payload" gene.

FIGURE 3 depicts transfection of an olfactory receptor neuron, and depicts the route, through the olfactory bulb, that the vector-encoded polypeptide will travel to reach and contact a terminus, located at the tip of a neuronal process which is part of a cholinergic neuron that has its main cell body inside the basal forebrain.

FIGURE 4 (which includes FIGS. 4A, 4B, and 4C) depicts the use of a genetic vector for the purpose of delivering anti-neurotrophic polypeptides to neurotrophic-factor-producing cells that reside wholly within the BBB, to reduce an unwanted excess of neuronal connections or activity, as occurs in conditions such as neuropathic pain. In this method, an "NPC" (neuropathic pain control) vector is used to transfect nocioceptive neurons which innervate tissues such as skin. The nocioceptive neurons will express vector-encoded polypeptides having anti-neurotrophic activity, and will release those polypeptides into spinal tissue. As depicted in FIGS. 4B (which shows abnormally large numbers of unwanted pain-signalling neuronal connections, before the treatment) and 4C (which shows a reduced number of unwanted pain-signalling connections, after the treatment), this type of blockade or inhibition of neurotrophic receptors or factors will lead to suppression or atrophy of the excessive neuronal connections that were causing or aggravating the neuropathic pain condition.

FIGURE 5 (which includes FIGS. 5A, 5B, and 5C) depicts the use of a genetic vector to transfect "spinal motor neurons" in a muscle for the purpose of delivering therapeutic polypeptides to a class of neurons (called "upper motor neurons") that lie wholly within the BBB. This type of treatment would be carried out, for example, in a patient with a limb that has become paralyzed or impaired due to stroke, injury, or disease. As shown in FIG. 5A, the gene vector will be injected into muscle tissue, where it will transfect spinal motor neurons that straddle the BBB; those neurons will express vector-encoded polypeptides having neurotrophic activity, and will release those polypeptides into spinal tissue, thereby delivering neurotrophic polypeptides to the upper motor neurons that are fully inside the BBB. FIG. 5B depicts a condition in a stroke-impaired or similar patient before treatment, in which some (but not all) of the neuronal "processes" which belong to upper motor neurons have degenerated and/or atrophied, and are no longer able to interact adequately with spinal motor neurons, leading to impairment or paralysis of a limb. FIG. 5C depicts an improved condition after treatment with a neuronal growth factor; in this condition, the still-functioning neuronal process which belongs to one of the

upper motor neurons has created ("sprouted") new and additional synaptic connections, which can interact with the spinal motor neurons that were not being adequately innervated prior to the treatment.

FIGURE 6 depicts the use of a genetic vector to transfect projections of lower motor neurons of the hypoglossal nucleus (a part of the brain stem), for the purpose of delivering therapeutic polypeptides through the BBB to neurons that reside wholly within the BBB. The exposed ends of these neuronal projections are present in the tongue; therefore, this class of neurons offers a relatively direct route for introducing foreign polypeptides into the brainstem portion of the CNS.

FIGURE 7 is a schematic display of the two ligatures that were emplaced around a sciatic nerve bundle in a rat, and of the phage-containing collagen gel that was emplaced in contact with the cut end of a sciatic nerve bundle, in a manner that enabled endocytotic uptake of phages into the sciatic nerve fibers.

FIGURE 8 is a photograph of fluorescent-labelled antibody-phage conjugates that accumulated within a sciatic nerve bundle, next to a hip ligature. The ligature (a loop of tightened suture material) prevented those antibody-phage conjugates, which had been internalised by the sciatic nerve fibers, from being retrogradely transported beyond the ligature constriction site.

FIGURE 9 schematically depicts a cyclic *in vivo* ligand selection process, in which ligand-displaying phages from a phage display library are selected for endocytotic uptake by nerve cells, using the *in vivo* method disclosed herein, and wherein the selected phage population that results from one cycle is used as the starting material for screening in the next cycle.

DETAILED DESCRIPTION

As suggested by the Summary section, above, this invention can be regarded has having four distinct elements or components. All four must work together, in a coordinated and sequential manner, in order to carry out the invention successfully. In a numbered and abbreviated summary, those four elements are:

A *(1) suitable genetic vector* carries a DNA sequence which includes at least one desired "passenger gene", which may include a marker gene (to simplify detection and analysis, during research) and/or a payload gene (which encodes a therapeutic or otherwise

desired and useful polypeptide). This type of vector is used to genetically transfect *(2) one or more selected types of neurons that straddle the blood-brain barrier* (examples include olfactory receptor neurons, and lower motor neurons). After the passenger gene has entered a BBB-straddling neuron, the neuron will transport the gene to the main body of the neuron, by a natural process called retrograde transport. After it reaches the main cell body, the gene will be expressed, thereby forming *(3) "foreign" or "exogenous" polypeptide molecules, inside the transfected BBB-straddling neuron.* These foreign polypeptide molecules (which, in some cases, may be identical to "endogenous" or "native" polypeptides that are not being made in sufficient quantities by a patient) will then be transported, still within the transfected BBB-straddling neuron, to a secretion site, located inside the BBB. The polypeptide molecules will then be secreted by the transfected BBB-straddling neuron, at the secretion site inside the BBB. This secretion will cause the foreign polypeptides to contact (and to treat, modulate, or otherwise affect, in a therapeutic or other useful manner) other types of *(4) "target" CNS neurons or other cells that lie entirely within the BBB.*

In the discussion immediately below, a virus-derived vector is used to illustrate this method of treatment; however, non-viral vectors alternately may be used, as discussed in later sections.

A first example which illustrates this method of treatment is illustrated in Figures 1 through 3, using consistent callout numbers in all three drawings. Figure 1 depicts this treatment on a "macroscopic" level; a liquid containing numerous copies of genetic vector 100 is introduced into the nasal sinuses of patient 80. The vector contacts the exposed "peripheral projection" 212 (see FIG. 3) of olfactory receptor neuron 200; these neuronal projections are accessible on the surfaces inside the nasal sinuses, and they can be directly contacted by various airborne compounds, as one of the biochemical processes involved in the sensation of smell.

As shown on a different scale (at a microscopic, cellular level) in FIG. 3, once the DNA carried by the genetic vector 100 has entered a neuronal projection 212, it is carried to the main cell body 224, by retrograde transport (discussed below). Because the genetic vector in this example has been derived from a type of virus that is fully capable of infecting nasal receptor cells, the vector DNA (which carries passenger gene 160, as shown in FIG. 2, as merely one component inserted into an disabled but infective virus genome) is able to help promote and facilitate this process. Accordingly, the use of infective viruses to

create the vector delivery system will help ensure that the transcribed portion of the passenger gene 160 will be expressed into messenger RNA strands 299 in at least some transfected neurons, as shown in FIG. 3.

The mRNA strands which have been transcribed from the passenger gene 160 will then be translated into multiple copies of foreign polypeptide molecules 300, by the normal cellular mechanism involving ribosomes 226 inside the transfected neuron 200.

Due to natural transport and secretion mechanisms (which can be enabled or enhanced, if desired, by using genetic engineering techniques as discussed below to add one or more specialized transport or secretion sequences to an end of a polypeptide chain), at least some of the foreign polypeptide molecules 300 will then be transported (using a process called anterograde transport) to various synaptic terminals 242 (or other peptide-secreting locations) that belong to the BBB-straddling neuron 200.

At these locations, which are located entirely within BBB-protected CNS tissue, the foreign polypeptide molecules 300 will be released by the BBB-straddling neuron 200. This secretion process allows the secreted foreign polypeptide molecules 300 to directly contact (and to begin exerting therapeutic or other modulating effects on) other classes of neurons which are referred to herein as "target" neurons; these "target" neurons lie wholly within the BBB. In the schematic illustrations shown in FIGS. 1 and 3, the polypeptides are secreted by transfected olfactory receptor neuron 200 within a roughly spherical globular structure called a "glomerulus" 910, which contains various types of "targeted" neuronal structures, as discussed below.

To condense this process into short form, this overview and FIGS. 1-3 indicate how *(1) a genetic vector* carrying a passenger gene, is used to transfect *(2) BBB-straddling neurons*, which will then express *(3) foreign polypeptides*, which will then be secreted into CNS tissue inside the BBB, where they will contact and affect *(4) "target" CNS neurons located wholly within the BBB.*

Each of these four elements are described in more detail below. Since the optimal design of a genetic vector will depend on the particular type of BBB-straddling neuron that will be transfected, BBB-straddling neurons are discussed first.

TYPES OF BBB-STRADDLING NEURONS

Neurons that straddle the BBB can be divided into three main classes: sensory neurons, motor neurons, and pre-ganglionic autonomic neurons. All of these classes of

BBB-straddling neurons discussed have been studied extensively, and genetic vectors are known that can be used to transfect each of these classes of neurons.

The first major class of BBB-straddling neurons addressed herein are the sensory neurons, which are the "front line" cells that are directly involved in sight, smell, taste, and various sensations involved in touch, pain, the sense of position of a limb, etc.

Sensory neurons have specialized components (referred to herein as "peripheral projections") that extend out to (or very near to) certain surface or tissue regions outside the BBB. As used herein, a "peripheral projection" is the portion of a BBB-straddling neuron that is most directly accessible to a genetic vector. A genetic vector will not need to cross the BBB, in order to contact a peripheral projection.

Conversely, the term "central projection" refers to a fibrous portion of a neuron which extends away from the main cell body, and which extends either: (i) through the BBB, if the main cell body is located outside the BBB, as occurs with many types of sensory neurons; or, (ii) closer to the center or the brain or the upper spinal cord, if the main cell body is located inside the BBB, as occurs with motor neurons and pre-ganglionic autonomic neurons.

The term "axon" also should be noted and understood, with regard to central or peripheral projections. In most cases, the term "axon" refers to the single largest and longest projection that emerges from a neuron's main body. However, many sensory neurons can be regarded as having two axons, with one extending toward the periphery, while the other extends toward the central portion of the brain. Because one of the primary roles of most BBB-straddling neurons is to shuttle nerve impulses between the peripheral and central nervous systems, the portion of a BBB-straddling neuron that actually crosses the BBB can almost always be properly classified as an axon.

Four classes of BBB-straddling neurons deserve particular consideration, for potential use as disclosed herein. Two of those classes are sensory neurons: (i) olfactory receptor neurons, and (ii) nocioceptive (pain-signalling) neurons. A third class comprises motor neurons, which are involved primarily with musculo-skeletal control; this class includes a subclass called tongue motor neurons, which are of special interest because their cell bodies are in the brainstem. The fourth class, called "pre-ganglionic autonomic neurons", are not highly promising candidates for most types of human medical uses, since genetic alteration of these neurons might affect the autonomic nervous system; however, they should be recognized, because they may be useful for treating certain types of medical

problems, and for various types of research. Each of these four subclasses is discussed separately, below.

Olfactory Receptor Neurons

One class of BBB-straddling neurons preferred for use herein includes olfactory receptor neurons. Because of the role they play in the sense of smell, their peripheral projections are exposed and accessible on the interior surfaces of the nasal sinuses, where they can be directly contacted and activated by various types of airborne molecules that are being drawn in through the nose.

These neurons are described in various textbooks on physiology, such as Guyton and Hall, *Textbook of Medical Physiology*, 9th edition (1996), pages 678-681, and additional references cited therein on pages 681-682. Very briefly, a human olfactory membrane has a surface area of about 2.5 square centimeters, and typically has about 100 million receptor neurons. The exposed tip of each olfactory receptor neuron usually has about 6 to 12 tiny hairs called cilia, which extend downward several microns into a layer of mucus. It is presumed that most molecules which trigger perceptions of odor interact with the cilia and/or receptor proteins that straddle the membrane of the exposed surface of a receptor neuron. Although most molecules which trigger sensations of smell do not enter the neurons themselves, it is known that a few specific types of molecules (such as wheat germ agglutinin, which binds in a non-specific manner to most glycoproteins) are taken up and transported into olfactory receptor neurons, presumably by some form of endocytosis. Several published reports have also stated that adenovirus vectors, when used to contact the olfactory membrane, can indeed transfect (i.e., insert foreign genetic material into) olfactory receptor neurons, as evidenced by subsequent expression of marker genes by the transfected neurons.

Olfactory receptor neuron 200, illustrated in a schematic manner in FIG. 3, comprises a number of cell parts that will be mentioned because of how they interact with one or more parts of the genetic vector 100. While the nasal sinus membrane 90 is shown schematically as a distinct layer, it is made up of the exposed apical surfaces of the olfactory support cells of the olfactory epithelium. A neuronal projection 210 passes through the nasal sinus membrane 90, creating an exposed terminus 212 (also called a mucosal tip, end, or knob). This exposed neuronal tip 212, which sits inside the nasal sinus cavity, allows the neuronal tip 212 to be contacted by genetic vectors (as disclosed herein)

that are carried by a liquid nasal spray.

Olfactory receptor neuron 200 also comprises cell cytoplasm 220, a nucleus 222 which sits inside the main cell body 224, ribosomes 226 which translate mRNA into polypeptides, and an axon 240 which passes through the blood-brain barrier (BBB).

As noted in the Background section, the BBB is not a single membrane structure; instead, it is a network of capillary walls that have unusually tight junctions between the endothelial cells that form the capillary walls. While some scientists might argue that the olfactory epithelium (i.e., the mucous membrane surface that contains the olfactory receptor neuron projections, inside the nasal sinuses) can be considered an extension of the brain, and therefore presents a region of CNS tissue where the BBB does not exist, it is clear from physiological studies that olfactory glomeruli sit entirely within the BBB, and are protected by the BBB from unwanted molecules that might trigger spurious and unhelpful nerve impulses. To represent that fact, double-dashed line 900 is used in FIG. 1 to schematically represent the BBB. The peripheral projection 210 and main cell body 224 of olfactory receptor neuron 200 sit outside the BBB, while axon 240 passes through it, and then branches into numerous synaptic terminals 242 which sit inside BBB-protected CNS tissue. Accordingly, olfactory receptor neuron 200 straddles the BBB.

The synaptic terminals 242 of olfactory receptor neuron 200 are located in a roughly spherical globular structure, shown as "glomerulus" 910 in FIG. 3. In a human, there are thousands of glomeruli, and each glomerulus contains the synaptic terminals of roughly 25,000 axons from olfactory receptor neurons. Each glomerulus 910 is also the terminus for thousands of dendrites and projections 920 from large "mitral" cells and smaller "tufted" cells. While the cell bodies and dendrites of the mitral and tufted cells are drawn as lying in the glomerulus, their main cell bodies are located in bulb structures positioned above the glomeruli. In addition, each glomerulus (or surrounding regions that are closely proximate to the glomerulus) also contains termini 934 located at the tips of long fibers (called processes) 932 which extend down from neurons 930, called "basal forebrain cholinergic neurons", since they are located in the basal forebrain, and since they are activated mainly by the excitatory neurotransmitter acetylcholine.

The schematic representations in FIG. 3 are selective illustrations, and do not attempt to illustrate all targeted neuronal structures. For example, also found in or in the near the glomeruli are termini of: (i) fibers extending from "serotonergic" neurons in a brain region called the raphe nucleus, which have their main cell bodies in a part of the

midbrain; and, (ii) fibers extending from "noradrenergic" neurons which have their main cell bodies in a part of the brain called the locus coeruleus nucleus. Depending on the therapeutic polypeptide being delivered, these or other neurons residing wholly within the BBB might be targeted by a treatment as disclosed herein.

In general, it is anticipated that polypeptides released by a transfected olfactory receptor neuron 200 are likely to be able to contact and exert modulating effects on any (or nearly any) type of neuron that has been shown, using so-called "trans-synaptic tracer studies" (e.g., Lafay et al 1991; Barnett et al 1993), to be infected by virus particles released by virus-infected olfactory receptor neurons. Such neurons include mitral cells and tufted cells (these are infected in large numbers, since very large numbers of their termini are present in the glomeruli), and at least some basal forebrain cholinergic neurons, raphe nuclei serotonergic neurons, and locus coeruleus noradrenergic neurons (which have also been shown to be infected, in tracer studies).

It should also be recognized that various types of "glial cells" are also likely to be contacted by polypeptide molecules 300 that are secreted by olfactory receptor neuron 200. As described in more detail below, glial cells (also called neuroglial cells) include various types of cells which cannot receive or transmit nerve signals, and which instead support and serve the neurons located inside BBB-protected CNS tissue.

Accordingly, the term "target cells" is used herein to refer to cells which sit entirely within BBB-protected CNS tissue, and which are the intended "targets" of the foreign polypeptide molecules 300 that are encoded by the passenger gene(s) in a genetic vector 100 as described herein. A BBB-straddling neuron which is actually contacted and transfected by a genetic vector is not regarded herein as a target cell; instead, that type of BBB-straddling neuron should be regarded as part of the delivery mechanism, and can be referred to by terms such as "delivery cell", "transfection conduit", or as a "primary" transfected neuron.

If olfactory receptor neurons are used as the delivery route, another physiological factor is potentially important, and should be recognized. Olfactory receptor neurons gradually die off, and are constantly being replaced by newly-created neurons. In mice, the "half-life" of olfactory receptor neurons is about 3 months, and the half-life in humans is presumed to be roughly comparable.

Therefore, if olfactory receptor neurons are transformed in a stable manner by genetic vectors (i.e., if the genetic vectors cause one or more foreign genes to be inserted

into the chromosomes of the olfactory receptor cells), the transformed cells will nevertheless gradually die off over the next weeks and months. As a result, depending on the type of vectors and polypeptides that are involved and the effects that are desired in a particular patient, it may be necessary to readminister additional vectors to the patient, every few weeks.

Nocioceptive Neurons

Another class of BBB-straddling sensory neurons transmits pain signals from the skin and body, into the spinal cord. These neurons generate nerve impulses in response to impinging substances, signals, or events from the environment; accordingly, these are the neurons that commence the process that generate feelings of pain or discomfort when the skin has been cut, scraped, or hit, or exposed to intense heat or cold. These pain-signalling neurons are usually called "nociceptive" or "nocioceptive" neurons, and are sometimes called nociceptors or nocioceptors. Nocioceptive neurons are just one of a number of different functional types of BBB-straddling sensory neurons that form part of the dorsal root ganglia.

FIG. 4 depicts, in a highly schematic fashion, a nocioceptive neuron 400. Like most other sensory neurons, this neuron 400 has its main cell body 402 in a tissue region which is outside of, and not protected by the BBB; in the case of nocioceptive neuron 400, the main body 402 is located relatively close to spinal cord 480, in a dorsal root ganglion. This neuron has a peripheral projection 410 which extends outwardly, i.e., away from the spinal cord 480, and toward the skin (other projections also extend to other regions deeper within the body). This peripheral projection 410 branches out into numerous "near-surface terminals" 412, located in shallow layers of tissue just beneath the skin surface 405.

In the other direction, nocioceptive neuron 400 also has an axon 420, which passes through the blood-brain barrier. As indicated in FIG. 4B, axon 420 branches into processes 422, 424, and 426 (shown in FIG. 4B), inside BBB-protected CNS tissue in spinal cord 480. Each process terminates in one or more synaptic junctions that allow the nocioceptive neurons to transmit its pain signal to "second order" neurons, inside spinal cord 480.

The surface of spinal cord 480, which is shown as a cross-sectioned segment, has: (i) a ventral median fissure 482 (also called an anterior median fissure), positioned toward the front of the patient; and, (ii) a dorsal median fissure 484 (also called a posterior median fissure), flanked by two smaller dorsal-lateral (or postero-lateral) fissures. The mass of

spinal cord 480 comprises "white matter" 486 which surrounds "gray matter" 490. The gray matter 490 comprises left and right ventral (anterior) horns 492, and left and right dorsal (posterior) horns 494. These four horns are all connected to a central portion, called the gray commissure. The large bundles of nerve fibers which emerge laterally from the spinal cord, as shown in FIGS. 4 and 5, usually are called the posterior (or dorsal) roots, and the anterior (or ventral) roots. The term "ganglion" generally refers to aggregations of neuronal cell bodies outside the CNS, and can also be used to refer to these nerve bundles.

If skin surface 405 is cut or scraped, the nerve signals that commence at the near-surface terminals 412 of nocioceptive neuron 400 travel through the peripheral projection 410, through the cell body 402, through the axon 420 which crosses the BBB, and into the branched dendrites 422-426 inside the dorsal horn 494 of spinal cord 480. The synapses at the tips of dendrites 422-436 release neurotransmitters, which trigger nerve impulses in the spinal neurons. Those nerve impulses travel up the spinal cord, to centers inside the brain which process the arriving nerve signals in ways that the brain interprets as pain.

Various medical problems have been grouped together under name, "neuropathic pain." As indicated by the name, *neuropathic* pain involves a pathological condition that affects neurons, in a manner that generates unwanted and excessive pain signals. This often involves some anatomical reorganization of the nerve connections within the BBB, such that there is a chronic and inappropriate pain response. The term "hyperalgesia" is also used, as a descriptive term that translates directly into "excess pain", and the term "allodynia" is also used for this condition.

Neuropathic pain is a well-known cluster of medical problems, and this broad category includes diabetic neuropathy, "phantom pain" from limbs or extremities that have been amputated, arachnoiditis, trigeminal pain, post-infective pain (such as outbreaks of "shingles", caused by herpes zoster viruses), and lingering chronic pain that arises after a traumatic injury or surgery and then will not recede, even after the normal timespan of recuperation has long passed. Causalgia is another type, and involves burning sensations (the root word "caus-" arises from the same root as "cautery", and has nothing to do with causation). Other types of neuropathic pain are also known, and it should be recognized that neuropathic pain can range over a very wide span of intensity, starting at annoying, up to excruciating, debilitating, and unbearable. Indeed, neuropathic pain is involved as a major factor in many suicides; chronic and incurable pain can be so intense and relentless that it drives many sufferers to commit suicide.

A cellular mechanism that is believed to be widely involved in many types or cases of neuropathic pain is illustrated in FIG. 4B. In this schematic drawing, axon 420NP has become involved in a neuro-pathological problem that has caused it to sprout too many dendrites 422, 424, and 426, which have begun to interact with spinal neurons 432, 434, and 436 (some of which may be inappropriate) located in the dorsal horn of spinal cord 480NP. This sprouting and chronic activation of too many dendrites from a single nocioceptor axon causes or aggravates the transmission of too many pain signals into and through the pain-plagued spinal cord 480NP. This condition, of too many neuronal connections involving pain-transmitting neurons, as shown in FIG. 4B, is referred to as "hyper-innervation".

This invention offers a method of controlling and reducing neuropathic pain, by administering a genetic vector that can transfect nocioceptor neurons in or near an affected area, as further described herein and illustrated in FIGS. 4A-4C. This type of vector, shown in FIG. 4A as vector 100NPC (the letters "NPC" refer to "neuropathic pain control"), will carry an "NPC" passenger gene which is designed to *suppress* (rather than increase) neuronal activity, as discussed below.

Since the nocioceptive receptors are likely to be located in shallow regions beneath the skin, subcutaneous, intramuscular, or other relatively shallow injection is a preferred route of administration. Alternately, topical and other modes of administration also can be evaluated, including: (i) topical application of a vector-carrying ointment, cream, or other solution or suspension, which can also contain an agent that promotes permeation through tissue, such as dimethylsulfoxide, methylsalicylate, etc.; (ii) topical application of a vector-carrying formulation to a roughened, abraded, or otherwise physically-treated area of skin; and/or (iii) topical application of a vector-carrying formulation to skin that has been chemically treated, such as by the types of chemicals that are used for "skin-peeling" treatments.

For purposes of further discussion, it is assumed that a liquid containing a genetic vector 100NPC, for neuropathic pain control, will be injected in a shallow subcutaneous manner into a region of skin at or near the location of an apparent "focal point" (also called a locus, seat, hot spot, etc.) where neuropathic pain is perceived most intensely by a patient. As mentioned above, vector 100NPC carries a gene designed to suppress (rather than increase) neuronal activity. Such suppressor genes can encode, for example: (i) monoclonal antibodies (or antibody fragments) that will bind to and inactivate one or more

types of neuro-trophic or other neuro-stimulatory peptides or compounds; or, (ii) peptide fragments that will competitively bind to, occupy, and block one or more types of neuronal cell receptors that are involved in neuro-stimulatory processes. The NPC gene will be transported (using retrograde transport, as noted above) to the cell body 402 of nociceptor neuron, where the gene will be expressed to form copies of an NPC polypeptide as described above. The NPC polypeptides will then be transported (using anterograde transport) by the neuronal axon 420, across the BBB and into a spinal cord 480NP which is being plagued by neuropathic pain, caused or aggravated by a hyper-innervation condition that has a component within the spinal cord, as shown in FIG. 4B.

The NPC polypeptides will be released into the BBB-protected spinal tissue, at or near the site of the hyper-innervation condition that exists inside the spinal cord. By exerting their suppressive effects (such as by binding to, blocking, competing against, or otherwise suppressing cellular agents or processes that stimulate or sustain higher levels of neuronal activity), the NPC polypeptides will help reduce and mitigate the neuropathic pain condition, either on a permanent basis, or on a basis that may last for days or weeks, depending on what type of treatment is used.

Motor Neurons

Another class of BBB-straddling neurons is usually called *motor neurons*. These mainly transmit instructions from the CNS to the muscles of the body, to "innervate" skeletal musculature and place the muscles under the control of the CNS. One major subclass of motor neurons, usually called "spinal motor neurons", have their cell bodies in the spinal cord, and projections that extend out through the BBB to contact peripheral nerve cells and muscle.

FIG. 5 (which comprises FIGS. 5A-5C) schematically depicts use of a spinal motor neuron as a "transfection conduit" (as described above) to stimulate and increase neuronal control over a muscle, in a patient who has a weakened limb due to a stroke, traumatic injury, neurodegenerative disease, or similar cause, by delivering therapeutic polypeptides to upper motor neurons that lie wholly within the BBB. FIG. 5A depicts spinal motor neuron 500, which has a cell body 502 located inside BBB-protected tissue in spinal cord 520. Spinal cord 520 has the same structure shown in FIG. 4A, and motor neuron cell body 502 is located inside the gray matter, in ventral horn 522. The axon of the spinal motor neuron 500 passes through the BBB, and forms a long "process" 504 which extends to

muscle fiber bundle 550. Inside the muscle fiber bundle 550, the neuronal axon or process 504 branches into dendrites and terminals 506, which (in a healthy person) interact with the muscle tissue to trigger muscle contractions at desired times, thereby providing "voluntary motor control" of the arms, legs, etc.

FIG. 5B is a schematic depiction of the cell bodies of three distinct spinal motor neurons, designated as 512, 514, and 516, in a patient who has suffered a stroke or a head or spinal injury, or who is suffering from a neurodegenerative disease (such as amyotrophic lateral sclerosis) or similar problem that has impaired his voluntary motor control over an arm or leg. This impaired condition is due, at least in part, to dead or damaged upper motor neurons in the brain or brainstem, above the location of the three motor neuron cell bodies. Some of the dead or damaged upper motor neurons in the brain or brainstem, above the location of spinal motor neuron cell bodies 512-516, had supplied nerve impulses to spinal motor neurons 512 and 516; as depicted schematically in FIG. 5B, when various upper motor neurons were damaged or killed, their processes 532 and 536 (indicated by dotted lines in FIG. 5B) fell silent and began to degenerate, leaving only one of the three spinal motor neurons (illustrated as the center process 534) with an active supply of incoming nerve signals, from its upper motor neuron. Since the two motor neurons 512 and 516 are no longer receiving any incoming nerve impulses, they have fallen into silence and disuse, and are in danger of atrophying, deteriorating, and dying over time.

FIG. 5C schematically depicts the result of treatment of this condition, following injection of a liquid containing copies of genetic vector 100 into the muscle bundle 550 that is no longer adequately functioning. Vector 100 carries a therapeutic gene which encodes a neurotrophic factor or other polypeptide that stimulates neuronal activity or replication. The gene carried by this vector is transported into the cell bodies 512-516, where the gene is expressed into neuro-stimulating polypeptide molecules. These polypeptides are then secreted by the three motor neurons, and they act as signals which stimulate and attract the growth and/or activation of additional dendrites from any nearby neurons which are still viable and active, including the upper motor neuron having the active process 534. This causes the active process 534 to sprout additional processes 534a and 534c, which can form synapses with other neurons. The additional newly sprouted synaptic junctions thereafter begin activating the processes from spinal motor neurons 512 and 516 once again. When coupled with a physical therapy and exercise program, this restores to the patient a greater degree of voluntary control over his arm or leg.

Tongue Motor Neurons

One subclass of motor neurons that may be highly useful in this invention has projections which extend through the BBB, to innervate the muscles of the tongue. These "tongue motor neurons" control the movement of the tongue, for both eating and talking. Their cell bodies are located within the brainstem; therefore, they offer a promising route for delivering foreign polypeptides into the brainstem portion of the CNS.

This is depicted in FIG. 6, which depicts a tongue motor neuron 600, having a cell body 602 located in the brainstem 950, and a long peripheral projection 604 which passes through the BBB and terminates inside the tongue 62 of patient 60. The accessible tip of neuronal projection 604 is contacted by a genetic vector 100A, by means of a carrier liquid injected into the tongue 62. The vector DNA will be retrogradely transported through the neuronal projection 604, into the cell body 602. The passenger gene will be expressed into a therapeutic polypeptide, which will be secreted by the tongue motor neuron 600, at locations in the brainstem. These secreted polypeptides will contact and exert their effects on various other neurons 952 and 954, located in brainstem 950.

PRE-GANGLIONIC AUTONOMIC NEURONS

The last major class of BBB-straddling neurons that will be specifically discussed herein is usually called *pre-ganglionic autonomic neurons*. Like motor neurons, their cell bodies are located inside the BBB. Their axons extend out through the BBB, and connect with nerves of the sympathetic and parasympathetic nervous systems. As part of the "autonomic" system, these neurons are involved in the control of various functions that are not under conscious control (such as blood pressure, digestion, excretion, sweating, etc.).

The term "ganglion" implies a bundle of neurons. Accordingly, "pre-ganglionic" neurons include neurons whose cell bodies are found within the CNS in clusters (also called nuclei) and whose axons project through the BBB to innervate and make contact with the neurons found in the ganglion lying outside the BBB.

It is believed that the methods and genetic vectors of this invention can be adapted and used, if desired, to genetically transfect pre-ganglionic autonomic neurons, and it is believed that in at least some cases, such transfected pre-ganglionic autonomic neurons will subsequently transport and deliver the foreign polypeptides into the brainstem and/or spinal cord, and possibly other CNS neurons lying wholly within the BBB (for generally supporting data, see, e.g., Pickard et al, 2002).

However, it should be recognized that sensory neurons and motor neurons are likely to be somewhat easier to work with and evaluate, at least during the early stages of development of this invention. This is due to several factors. On one hand, the projections of sensory and motor neurons actually reach (or closely approach) exposed and accessible surface locations (in the case of olfactory neurons), and relatively shallow muscle and subcutaneous regions (in the case of pain-transmitting and motor neurons). By contrast, pre-ganglionic autonomic neuron terminals are buried substantially deeper beneath the skin surface, within the autonomic neuronal ganglions. Therefore, pre-ganglionic autonomic neurons are likely to pose somewhat greater technical challenges (and somewhat greater risks) for accurate vector delivery than sensory or motor neurons, if used as BBB-straddling "transfection conduits".

Therefore, the examples and most of the discussion herein focus on using sensory neurons or motor neurons, as BBB-straddling neurons that can be transfected by genetic vectors, to describe and illustrate the invention herein. Those classes of BBB-straddling neurons are believed to provide generally preferred candidates for initial development and testing of this invention. Nevertheless, pre-ganglionic autonomic neurons should be recognized as having potential use as BBB-straddling "transfection conduits" as disclosed herein, and may eventually become highly useful for various types of therapeutic or other treatments.

GENETIC VECTORS

As summarized above, genetic vectors (such as viral vectors, liposomes, and ligand vectors that target endocytotic receptors on BBB-straddling neurons) that carry one or more useful "passenger" genes (which can include marker genes and/or payload genes) are contacted with the peripheral projections of BBB-straddling neurons, in a manner which promotes transfection of vector DNA (including the useful passenger gene) into one or more BBB-straddling neurons. Once inside such neuron(s), at least some copies of the passenger gene(s) will be transported through the projection to the main cell body, by the natural process of retrograde transport. Once inside the cell body, the passenger gene(s) will be expressed by the normal intracellular mechanism, to create the gene-encoded polypeptide.

Since most polypeptides of interest herein (such as neurotrophic growth factors) act as hormones or growth factors, which normally and naturally must be secreted by cells in

order to carry out their necessary functions, such polypeptides, when expressed by transfected BBB-straddling neurons, will be well-suited for secretion by the transfected neurons.

Accordingly, this invention discloses a class of genetic vectors that can be used to transfect certain types of selected CNS neurons which straddle the BBB, in a manner which causes the transfected neurons to express and then secrete useful and therapeutic polypeptides, into CNS tissue that normally is protected from foreign polypeptides by the blood-brain barrier.

FIG. 2 shows a schematic depiction of a viral vector 100. This vector can be regarded as having three primary components: an encapsulating portion 110, binding ligand proteins 120, and genome 150 (which, in FIG. 2, is shown as double-stranded DNA, or dsDNA).

In viral vectors (such as vectors derived from adenoviruses) that do not have lipid "envelopes", the encapsulating portion 110 is made of capsid proteins 112, which fit together in a semi-interlocking manner. In such vectors, the protein "shell" is usually called a capsid, and the binding ligand proteins 120 usually are nothing more than capsid protein domains which are exposed on the exterior surface of each viral particle.

In other viral vectors (such as vectors derived from herpes viruses), the encapsulating portion (usually called an "envelope") is made of lipids, usually arranged in a bilayer form which is comparable to the lipid bilayers that make up the outer membranes of most mammalian cells. In such vectors, the binding ligand proteins 120 usually straddle the envelope layer, and a protruding external portion of each protein extends outwardly, so it can contact and bind to cell surface proteins.

Regardless of whether a lipid envelope is present, the viral binding ligands 120 adhere (in a non-covalent manner, comparable to a binding reaction between an antibody and an antigen) to complementary proteins on the surfaces of cells that can be infected by that type of virus. Some types of viruses (and vectors derived from them) can have binding ligands that are highly specific; these types of viruses can infect only certain types of cells which have complementary surface proteins. Other types of viruses (and vectors derived from them) have binding ligands that are much less specific, and can bind to and infect a much wider variety of cells.

The vector genome carried by the vector 100 shown in FIG. 2 comprises double-stranded DNA (dsDNA). Other types of viral vectors can carry single-stranded DNA, or

single- or double-stranded RNA. Various genetic vectors with all four categories of genomes are known, and any such vector can be evaluated for use as disclosed herein. In general, viral vectors carrying dsDNA genomes are regarded as likely to offer preferred candidates for early evaluation of this invention. During laboratory manipulation of synthetic or isolated genes, it tends to be easier to work with dsDNA, than with ssDNA (which tends to be "sticky" due to the innate attraction of the bases for each other) or RNA (which tends to be somewhat less stable than DNA). Accordingly, most of the viral vectors that have been developed to date (including vectors derived from herpes viruses or adenoviruses) contain dsDNA genomes.

Using conventional terminology that has previously been developed for use with viral vectors, genome 150 carried by a viral vector (and by various other types of genetic vectors as well) can be regarded as comprising a number of "domains". One or more "passenger genes" 160 will most commonly be inserted somewhere into the middle of the viral genome, in order to ensure that both ends of the viral genome can function properly once the viral genome enters a transfected cell. Accordingly, the insertion of passenger gene 160 into the center of a viral genome results in creating two "flanking sequences" 155 and 157, containing native or modified viral DNA sequences which flank both ends of the passenger gene. The tips of the flanking sequences 155 and 157 frequently will be coupled to viral polypeptides 158 and 159; in general, these types of polypeptide "caps" evolved to help viral DNA remain relatively stable and resistant to the defensive mechanisms inside a cell that normally attempt to chew up and dismantle viral DNA after it has invaded a cell. If desired, either or both these polypeptide "caps" can be selected and/or modified to promote and speed up various cellular processes (such as retrograde transport, active transport of the viral DNA into the cell nucleus, etc.).

The "passenger gene" 160 can also be regarded as having three distinct domains. The promoter region 162 contains a signalling sequence, which directs "transcriptase" enzymes to get ready to begin transcribing that strand in the DNA double helix, to form strands of messenger RNA. This promoter region normally contains a so-called "TATA" box or similar signal, which directs the enzymes to begin transcribing mRNA from the DNA sequence, at a location which is usually about 25 bases downstream from the TATA box. For simplicity of discussion herein, that sequence of about 25 bases, located between the TATA box and the first base which is transcribed into mRNA, is regarded herein as part of the gene promoter. Alternately, if preferred, it can be referred to as part of a "non-

transcribed leader sequence", which would not need to be regarded as part of the actual gene promoter.

The gene promoter is then followed by a "coding" region 164, which determines the sequence of the bases in the mRNA strand that will be transcribed from that gene. This region includes the DNA sequence that specifies an AUG "start" codon in the mRNA strand (which specifies a methionine residue as the first amino acid at the N-terminus of the polypeptide), and a "stop" codon (which truncates the translation of the mRNA strand by the ribosomes). In some vectors, the coding region in a passenger gene may also contain introns, which will be deleted from the final mRNA strand by "editing" processes inside the host cell.

The coding region is then followed by a "non-translated" sequence 166. This domain will be transcribed and will be part of the mRNA strands that are created inside a host cell. These domains, when present on mRNA strands, help stabilize the mRNA strands inside host cells, and protect them against rapid degradation by enzymes. However, this non-translated sequence is not translated into a polypeptide sequence, by ribosomes.

Variations on this general genetic structure are possible and may be used in this invention. As just one example, after the coding region 164 for one polypeptide and before the non-translated sequence 166, an "internal ribosome reentry site" (IRES), such as derived from a RNA virus such as encephalomyocarditis virus, may be inserted with a second coding sequence to allow co-expression of a second polypeptide (e.g., Wong et al 2002). The IRES instructs ribosomes to bind to the mRNA segment in a second location, and to commence expressing a second polypeptide in parallel with the first.

Viruses have evolved various different ways of introducing their DNA (or RNA) into cells, and genetically engineered viral vectors can make use of the same types of infective processes to deliver their DNA "payload" into susceptible cells.

As one example, in a process that is fairly common process among viruses that do not have lipid envelopes, a virus's capsid proteins will attach to protein molecules that are displayed on the host cell's outer membrane. This attachment process initiates a process called "invagination", which results in the virus being drawn into and becoming packaged within a lipid bilayer envelope, or vesicle, that is formed entirely of lipids from the cell's membrane, but which is now located inside the cell, suspended in its cytoplasmic fluid. This bubble-like vesicle, often called an "endosome," subsequently ruptures, either with the aid of the virus capsid proteins, or due to digestive processes or organelles inside the cell's

cytoplasm. This rupturing of the endosome liberates the viral DNA into the cell's cytoplasm.

In another example, in a process which is fairly common among herpes viruses and other viruses that have lipid bilayer envelopes, the different lipid bilayer membranes which enclosed the virus and the cell become fused together, and effectively merge with each other. This results in the entire contents of the virus's lipid envelope being transferred into the cell cytoplasm.

Regardless of which infective method is used by a selected type of viral vector, the result is that an effective viral vector transfers some or all of its genetic material into the cytoplasmic fluid of a cell. Returning to the example illustrated in FIG. 1, viral vector 100 inserts its DNA 150 into the exposed mucosal tip 212 of olfactory receptor neuron 200.

In some cases, after a genetic vector carrying a passenger gene has transfected neurons which straddle the BBB, any of several other fates and effects can result, depending on the replication and transmission traits of the vector system that was used. In most cases, it is assumed that the vector and passenger genes will remain inside transfected BBB-straddling neurons, and polypeptides expressed by the passenger genes in those transfected neurons will simply be secreted by the transfected neurons. However, some types of vectors (referred to as "trans-neuronal" vectors) may themselves be secreted by the BBB-straddling neurons, at locations inside the BBB; this would allow such trans-neuronal vectors (or their genetic material) to contact and transfect other neurons which are entirely inside the BBB.

It also should be noted that genetic vectors and gene constructs developed for use as disclosed herein can use any of various techniques and DNA sequences that are known for expressing higher quantities of an encoded polypeptide. Several such techniques and sequences are discussed below, under the subheading, "Gene and Vector Constructs; Expression, Transport, and Secretion Enhancers".

It also should be recognized that "marker genes" (also called "reporter genes"), which can allow easier, faster, less expensive, more reliable, or otherwise enhanced detection, quantification, and/or isolation of transformed cells and cellular pathways and fates, offer an important category of passenger genes which can be carried by genetic vectors as disclosed herein. Such genes, which are well known in genetic engineering, can allow facilitated and improved research, evaluation, and development in various fields of industry, commerce, and medicine, in various ways that will be readily apparent to those

skilled in the art.

Additional comments on various types of genetic vectors are contained in the Examples, below.

CANDIDATE THERAPEUTIC POLYPEPTIDES

The genetic vectors disclosed herein should be regarded essentially as vehicles, carriers, or delivery systems. As such, the particular choice of passenger and payload molecules that can be transported and delivered, by these vehicles, is largely up to the choice of a particular user who intends a particular use.

As a general rule, the genetic vectors disclosed herein will be able to transport and deliver essentially any chosen gene, having any nucleotide sequence, in a manner that will allow the corresponding polypeptides to be expressed by that gene, in transfected neurons. As such, most polypeptides that will be of interest to most physicians and researchers will be polypeptides that can be secreted, by the transfected BBB-straddling neurons, at sites located within the BBB. However, the requirement for secretion, in most cases that will be of interest to physicians and researchers, should not be regarded as a severe limitation, for two reasons. First, nearly all types of "neuroactive" polypeptides are inherently secreted; as a general rule, their neuroactivity was discovered and recognized due to their ability to be secreted by one type of cell within the CNS, and to act upon other types of cells within the CNS. And second, if some particular candidate polypeptide that is of interest is not naturally and normally secreted by cells, that polypeptide sequence can be coupled to any of numerous known "leader" polypeptide sequences that cause cellular secretion.

It should also be recognized that the endocytotic ligands disclosed herein do not address and are not directly concerned with secretion of polypeptides or other compounds, by transfected neurons. Instead, these ligands are designed and intended to enable targeted delivery of gene vectors to selected BBB-straddling neurons. It also should be noted that these same types of endocytotic ligands can also function as transport and delivery systems for other classes of passenger molecules, including therapeutic drugs, diagnostic compounds, or other compounds, into BBB-straddling neurons (and other classes of targeted cells) having endocytotic surface molecules to which the ligands will specifically bind).

Therefore, it may be that in some cases, drugs or other non-DNA compounds that can be transported into BBB-straddling neurons, by means of entry through peripheral projections that are accessible outside the BBB, may well be secreted by those same

neurons, at central projections that are located inside the BBB (as has been demonstrated with certain trans-synaptic tracer molecules, such as wheat germ agglutinin).

This route of delivery for drugs and other compounds may be especially promising, if the coupling component used to connect the passenger component to the endocytotic ligand can enable the passenger to be detached from the ligand, after the ligand-passenger complex has entered a BBB-straddling neuron. Such coupling components are often referred to as "labile" agents, which indicates that they are not especially tight or durable, and can be broken apart under various conditions. Various types of labile coupling components are known to those skilled in the art (the acidity-sensitive spacer molecules described in US patents 4,631,190 and 5,144,011, by Shen et al, offer one example). Any such labile coupling component can be evaluated, if desired, to determine whether it can be used with a particular type of therapeutic drug, analytical compound, or other passenger component that is of interest, in conjunction with a particular endocytotic ligand (or class of ligands), to form molecular complexes that can: (1) deliver the passenger components into BBB-straddling neurons, via accessible peripheral projections, and (2) enable the passenger components to be detached from the endocytotic ligands, after the complex has entered a BBB-straddling neuron, in a manner that (3) will allow secretion of the released passenger component, by the BBB-straddling neuron, at a secretion site located inside the BBB.

Returning to the issue of the range and variety of polypeptides that can be introduced into CNS tissue by using genetic vectors to introduce foreign genes into BBB-straddling neurons as disclosed herein, Tables 1 and 2 list some of the options that are available in just one area of application (i.e., in therapeutic treatment of various neurodegenerative or neuropathic diseases, in humans). To provide additional information on items that are listed without any narrative support in the Tables, the following summary and overview provides a somewhat expanded listing of various types of polypeptides that can be used in human medical therapy, and of the specific classes of disorders that the various polypeptide types can modulate. This discussion is not exhaustive, and those skilled in neurology or neuropharmacology will recognize other potential uses and therapies, after the methods disclosed herein have been made known to the public.

1. Various neurotrophic factors, growth factors, or neurite inhibitory factors, such as listed in Table 1, may help prevent or repair various forms of neuronal damage caused by CNS disorders such as neurodegenerative diseases, or by ischemic or hypoxic crises such as stroke, cardiac arrest, suffocation, blood loss, or other types of physical injury or

trauma.

2. Various neurotrophic hormones, growth factors, or neurite inhibitory factors can help stimulate the formation of new synaptic connections between existing neurons and/or guide the outgrowth of neuronal processes to facilitate some connections and discourage others. In some patients, this type of treatment can help facilitate the recovery of nervous function loss due to aging or various diseases. It may also help patients regain muscular, speech, and other functions after a stroke, head injury, or other ischemic, hypoxic, excitotoxic, or similar crisis.

3. Various types of endocrine, paracrine, and related or similar polypeptides can help treat various glandular, growth-related, maturation-related, sexual, and other disorders.

4. Polypeptides that can increase the quantities of certain neurotransmitter molecules inside the BBB can treat various neurodegenerative diseases. For example, polypeptides that can increase dopamine levels inside the brain (by acting as enzymes, hormones, or release factors, or through various other mechanisms) can be used to treat Parkinson's disease. Alternately, polypeptides that can increase acetylcholine levels may be useful for treating Alzheimer's disease.

5. Cytotoxic or growth-suppressing polypeptides can be used inside the BBB to treat certain types of cancer or other diseases.

6. Various types of receptor antagonists, antibodies, and other polypeptides that can block or suppress one or more types of neuronal activity can be used to help control and reduce neuropathic pain, hyperalgesia, and similar problems.

7. Lysosomal storage diseases due to lack of a particular polypeptide in the CNS may be treated by delivery of that polypeptide into the CNS.

8. Infections of the CNS by viruses, prions, or bacteria may be treated by delivering into the CNS that help control or reduce the spread of the infection. For example, delivery of polypeptides that bind to the receptors and inhibit virus docking may be able to reduce the spread of viruses such as HIV within the CNS.

9. Delivery of recombinant antibodies to antigens within the CNS can be used to modulate physiological processes in a beneficial or useful way. For example, delivery of recombinant antibodies to myelin associated neurite inhibitory molecules such as No-Go may be able to enable regrowth and regeneration of CNS nerves, following spinal cord injury and other traumatic injuries.

TARGETED CNS NEURONS OR GLIAL CELLS

The term "target cell" is used to refer to a neuron or glial cell that: (i) is part of the CNS, and lies wholly within the BBB; and (ii) is contacted by, or is intended to be contacted by, an exogenous polypeptide that has been delivered into BBB-protected brain or spinal tissue by a method and vector as disclosed herein.

It should be noted that this term deliberately excludes BBB-straddling neurons, even though such neurons can be regarded and referred to by terms such as "primary" or "initial" targets of the genetic vectors that will be used to contact and transfect such cells. In the overall scheme of the invention disclosed herein, the transfection of such BBB-straddling "initial targets" will be valuable and useful, only insofar as that step in the multi-step process will later lead to the subsequent delivery of vector-encoded polypeptides into CNS tissue that is protected by the BBB. Accordingly, the real "targets" of this invention are neurons or glial cells that are entirely within, and protected by, the BBB, and the BBB-straddling neurons that are used in the delivery mechanisms disclosed herein should be regarded as conduits, or passageways, rather than as the real target cells.

Any references herein to "glial" cells arises from the fact that, within BBB-protected brain and spinal tissue, cells are divided into two categories, referred to as neurons, and glial cells (also called "neuroglia cells" in some medical texts). By definition, the term "neuron" is limited to cells that can receive and transmit nerve signals. The term "glial cells" is a broader residual term, and it includes all types of CNS cells that cannot receive or transmit nerve signals. These glial cells perform various activities that can be regarded as supporting, housekeeping, and "nursing" functions within the CNS; this helps neurons do their essential work. The word "glia" comes from the same root word as "glue"; glial cells were initially thought of as the "glue" that holds CNS tissue together. Glial cells are divided into various categories, including oligodendroglia cells, astrocytes, ependymal cells, and microglia cells. They are discussed in nearly any textbook on neurology, and are a crucial part of the CNS.

For several reasons, the initial work to develop this invention is likely to focus upon using exogenous polypeptides to contact and modulate neurons, rather than glial cells. One major reason driving that trend is that it is likely to be much easier to confirm and quantify CNS responses that directly involve neurons, as compared to effects and responses that involve glial cells first, and affect neurons only as a secondary effect.

Accordingly, while some researchers will prefer to evaluate this invention by

focusing on "target cell" neurons lying entirely within the BBB, it should be recognized that: (1) the methods and vectors of this invention may also be useful for providing ways to treat CNS tissue by using exogenous polypeptides to contact and treat glial cells; and (2) certain specific conditions involving glial cells are likely to merit relatively early research and evaluation, such as potential treatments for certain types of glial cell cancers (including glioblastomas and astrocytomas), and methods for modulating the responses of glial cells to traumatic injury, or hypoxic or ischemic insult.

As implied by the term "target", not all neurons or cells wholly within the BBB will necessarily make contact with polypeptides delivered using the invention. Where a BBB-straddling neuron (or cluster of neurons) is transfected by a genetic vector, the "target" neurons or glial cells lying wholly within the BBB will be either: (i) positioned in close proximity to the BBB-enclosed synapses or other terminals of transfected neurons; or, (ii) have cell processes or extensions (such as dendrites, axons, or terminals) that are in close proximity to the BBB-enclosed synapses or other terminals of transfected neurons. Where a "transneuronal" vector is used (i.e., where the genetic vector itself will be able to travel through a BBB-straddling "primary" neuron, to a second- or third-order neuron that is located inside the BBB, and that will be transfected by the vector so that it will subsequently express the encoded polypeptide), "target" neurons or glial cells may be located in close proximity to second-order or third-order transfected neurons which will express and secrete polypeptides encoded by vector-borne foreign genes.

As examples of how one or more classes of neurons inside the brain can become "target" neurons inside the BBB, basal forebrain cholinergic neurons, serotonergic raphe neurons, and noradrenergic locus coeruleus neurons do not have direct synaptic junctions with BBB-straddling olfactory receptor neurons; nevertheless, these classes of cholinergic, serotonergic, and noradrenergic neurons inside the BBB can be target neurons, if olfactory neurons are transfected by a genetic vector as disclosed herein, because they can take up polypeptides released by transfected olfactory receptor neurons. This is illustrated by the fact that these classes of cholinergic, serotonergic, and noradrenergic neurons inside the BBB can be infected by rabies or herpes simplex viruses released from virus-infected olfactory receptor neurons (Lafay et al 1991, Barnett et al 1993).

The principle that CNS neurons or glial cells can be "targeted" for contact and treatment by exogenous polypeptides, if they are positioned inside the BBB adjacent to or within fairly close proximity to a central projection of a BBB-straddling cell, can be used

most advantageously if supported by the best currently available information on the anatomical relationships between transfected and targeted cells. This type of anatomical knowledge can be found in the technical literature where investigators have made use of viruses or other "transneuronal labelling" molecules. For example, if a particular class of CNS neuron or glial cell has been shown to become infected with a transneuronal virus, following application of that type of virus to BBB-straddling neurons in lab animals, the subsequently-infected class of CNS neurons or glial cells can be used as target neurons or glial cells for the purposes of this invention. This type of neuronal mapping has already been carried out to some degree by various researchers, and is described in references such as Loewy 1998 and Norgren et al 1998. In addition, this type of work continues to this day, and as it continues to provide more information on neuronal circuitry, that additional information can be taken into account by anyone practicing this invention.

The preferred method for contacting a genetic vector with a peripheral projection of a sensory or motor neuron will depend on the structure and location of the targeted peripheral projection. For example, administration to olfactory receptor sensory neurons can be via nasal instillation, such as by using a nasal spray, or by using a liquid-saturated packing material that can be placed in the nasal sinuses, in direct contact with the nasal surface area which contains olfactory projections, for some period of time (such as 30 to 90 minutes).

If desired, direct and sustained contact between a gene vector and the olfactory neuron projections can be further promoted by steps such as (i) using a nasal decongestant to reduce and minimize any mucous covering the nasal sinus surfaces; (ii) using a preparation of a cleaning or similar agent, such as dilute isopropyl alcohol, acetone, etc., to further clean and prepare the area to be contacted; and/or (iii) using a mechanical scraping procedure, as is commonly performed by otolaryngologists to treat patients with recurrent nasal sinus infections. In general, neuronal projections in a surface area which has become irritated to a point of mild inflammation tend to be more receptive, to cellular uptake of foreign molecules, than cells which can be regarded as being in a quiescent or resting state. It may also be possible to further stimulate entry of genetic vectors into neuronal projections by means of an electrical charge or surge applied to the nasal surface, in a manner comparable to the *in vitro* cell transfection technique of "electroporation". If desired, any or all of these steps can be carried out while a patient is under general anesthesia, or under a mild local anesthesia.

If desired, neuronal contact and uptake can be further promoted and increased by compounds referred to as "muco-adherents". Such compounds are being developed to increase the delivery of various drugs into the bloodstream, via transmembrane modes such as nasal sprays. Examples of muco-adherents include chitosan (discussed in articles such as Schipper et al 1999) and various polysaccharide colloidal preparations (discussed in articles such as Janes et al 2001); also see Rillosi et al 1995 and Lim 2000 for further discussion of muco-adherents.

Administration to projections which are part of nocioceptive neurons can be via cutaneous or sub-cutaneous injection, by various controlled skin abrasion techniques, and possibly by topical application if adequate penetration can be achieved (skin penetration can be promoted by agents such as dimethyl sulfoxide, if desired). If motor neuron projections are used as the access route, administration usually will require sub-cutaneous or intramuscular injection. For example, projections which are part of the lower motor neurons of the hypoglossal nucleus can be accessed by injecting a genetic vector into the muscles of the tongue. In some cases, gene vectors may be administered via an intravenous route (especially if a particular nerve-targeting component is included); however, it is generally believed preferable to administer gene vectors by intramuscular or similar injections that will establish and sustain high concentrations of vectors at desired targeted locations.

This also points out a significant difference between this mode (involving targeted transfection of selected neurons), and delivery of genes, polypeptides, or other compounds via injection into cerebrospinal fluid. Physical delivery into the cerebrospinal fluid system (such as via catheter into the ventricles of the brain or intrathecal space of the spinal cord) will distribute a neurologic agent to a large number and array of CNS cells. In contrast, this invention describes how delivery of desired therapeutic polypeptides can be targeted to, or result in controlled and preferential delivery to, only limited number of cells or neuronal processes, which are in close contact with (or synapsing with) transfected BBB-straddling neurons.

It is anticipated that the same procedures and classes of genetic vectors disclosed herein can be adapted and used, if desired, to introduce polypeptides into the brains and spinal cords of non-human mammals, and into other classes of animals that have blood-brain barriers, including reptiles and birds. As such, this invention may well become useful for controlling and regulating the rate of growth and/or reproductive status of livestock,

pets, and other animals. For this and other purposes, hypothalamic releasing factors can be delivered via the olfactory receptor neurons to regulate the release from the pituitary gland of potent hormones. For example, GHRH may be delivered to stimulate release of growth hormone, to accelerate an animal's growth rate. Also, sustained delivery of GnRH at supramaximal dose may be used to inhibit normal release of LH and/or FSH release, in a manner which may achieve or promote contraception, including reversible contraception. If the methods disclosed herein are being adapted to other mammalian species, or to other classes of animals such as birds and reptiles, the primary adaptations that will need to be made include (i) selection and use of genetic vectors that are well-suited for transfecting the projections of BBB-straddling neurons in the chosen animal type, and (ii) selection and use of a polypeptide type which has the desired activity in that particular species of animal.

Without disregarding these and other potential applications, the remaining discussion herein of genetic vectors focuses solely on animal tests to prove the methods and procedures disclosed herein, and on treating humans for medical purposes.

VIRAL VECTORS

Review articles that describe various types of mammalian viral vectors include Karpati et al 1996, and Kaplitt and Makimura 1997. At least four types of viral vectors have been used to transfect neurons. Those virus types are:

A. Adenoviruses

These are double-stranded DNA viruses that are often found in various glands; wild-type viruses cause respiratory infections, conjunctivitis, and various other problems. Genetically-engineered adenoviruses that have been rendered incapable of replicating (except in special types of cells and/or culture media that exist only in laboratory conditions) have become the main class of viral vectors used in *in vivo* studies on mammals, including gene therapy efforts on humans. Adenovirus vectors have been used to transfect various types of neurons, as reviewed in Smith and Romero 1999.

B. Adeno-Associated Virus (Dependovirus; adenosatellite virus)

These are single-stranded DNA viruses that depend on Adenoviruses for replication. Methods for preparing adeno-associated virus vectors can be found in the chapter by Bartlett and Samulski, in Robbins (ed.) 1997.

C. Herpes Simplex Viruses (HSV)

These are double-stranded DNA viruses, with capsids that are surrounded by lipid envelopes. Use of engineered HSV vectors to transfect neurons is discussed in Staecher et al 1998.

D. Retroviruses, including Lentiviruses

These viruses contain RNA, rather than DNA. Use of a lentivirus vector to deliver and express the GDNF gene into lower motor neurons of mice was described in Hottinger et al 2000.

These four classes of viruses appear to be receiving the most effort and attention at this time, in attempts to create viral vectors that can transfect cells but which are generally nonpathogenic after they enter their target cells (usually due to deletions or defects in one or more genes which encode proteins required for replication).

However, other classes of known neurotropic viruses (which includes numerous types of viruses that cause viral encephalitis, including some RNA viruses) offer promising candidates for vectors that can selectively (or at least preferentially) transfect neurons. If any of those classes of viruses can be rendered safe and non-pathogenic by means of genetic manipulation comparable to the steps used to render other viral vectors non-pathogenic, they may be well-suited for use as disclosed herein.

Alternately or additionally, using genetic engineering techniques, various types of viral vectors that do not have a strong affinity for neurons can be provided with selected and/or modified surface proteins (including chimeric surface proteins) that bind preferentially to surface proteins on the projections of olfactory neurons, motor neurons, or other types of neurons that straddle the BBB. If properly developed and used, modified viral surface proteins with increased neuron-binding affinity may increase the speed, efficacy, and other benefits of viral vectors when used as disclosed herein.

NON-VIRAL VECTORS

As briefly summarized in the Background section, and as discussed in more detail in various examples below and in numerous published articles, several types of non-viral genetic vectors are known, and can be evaluated for use as disclosed herein. Primary candidates for classes of nonviral vectors which can transfect neuronal projections include

(1) cationic materials, including cationic liposomes, and (2) protein-DNA complexes containing polypeptide ligands that bind to endocytotic receptors on neurons.

On the subject of ligands that can bind to endocytotic receptors on neurons, it should be noted that a highly useful and potentially very powerful *in vivo* screening method has been developed by the Applicants herein, for identifying and isolating ligands which can bind to endocytotic receptors on the surfaces of neuronal fibers outside the BBB. Briefly, this new method of *in vivo* screening uses the following steps:

(1) emplacing multiple candidate ligands at a first location inside the body of a living animal, in a manner that causes the candidate ligands to directly contact neuronal fibers (such as a sciatic nerve bundle, in the leg of a rat);

(2) allowing enough time to pass for the neuronal fibers to internalise those particular ligands that activate and drive the process of endocytotic internalisation and retrograde transport;

(3) harvesting segments of the neuronal fibers, at a harvesting site that is sufficiently distant from the ligand emplacement site to avoid collecting ligand candidates that did not enter the neuronal fibers or that were not retrogradely transported; and,

(4) removing the ligands from the harvested segments of neuronal fibers, for reproduction and further processing.

This new method of *in vivo* screening offers a powerful tool for developing improved non-viral vectors (and targeted viral vectors as well) that can bind specifically to any particular endocytotic receptor or other endocytotic surface molecule. Accordingly, since it offers a preferred mode of carrying out this invention, it is described in detail herein, under a separate subheading and in various Examples, below.

It should also be recognized that numerous methods and tricks are known to those skilled in the art, for increasing the likelihood that a non-viral vector will succeed in accomplishing its intended results. As just one example, a neuron-targeting non-viral vector has been reported which makes use of neurotensin, to target plasmid delivery to neurons of the nigrostriatal and mesolimbic dopaminergic systems (Martinez-Fong et al 1999). Various other examples are discussed in the following sections.

In general, plasmid vectors constructed for use with a cationic or endocytotic delivery mechanism will contain both: (i) sequences to enable replication of the plasmid in a host cell, such as *E. coli*, and (ii) sequences that enable expression of the gene for a therapeutic polypeptide in target cells. For replication, the vector ordinarily carries a

replication site, as well as marker genes that allow simple selection of transformed cells. For example, for replication in *E. coli*, the plasmid pBR322 (Bolivar et al 1977) contains genes for ampicillin and tetracycline resistance, which allow quick and simple identification of transformed cells by using those antibiotics.

The sequences that enable expression of the gene for a therapeutic polypeptide in a target cell ordinarily include an origin of replication (if necessary), a promoter located in front of the gene to be expressed, any necessary ribosome binding sites and/or RNA splice sites, a polyadenylation site, and transcriptional terminator sequences.

GENE AND VECTOR CONSTRUCTS; EXPRESSION, TRANSPORT AND SECRETION ENHANCERS

A variety of known genetic engineering techniques and DNA or polypeptide sequences can be used to improve and increase: (i) the likelihood that this method will successfully accomplish a detectable level of a desired and intended result, in any particular animal or patient; (ii) the potency, efficacy, duration, or other desired aspects of the treatment, in treated animals or patients; and, (iii) the ability of researchers and physicians to track and monitor the status, progress, and results of a treatment, and the locations and concentrations of exogenous genes and/or polypeptides.

This section describes various examples of such known techniques and sequences, along with a brief indication of how they can be applied to the methods and vectors of this invention. This listing of illustrative examples is not exhaustive or exclusive, and those skilled in the art will recognize various other genetic engineering techniques, reagents, and gene and peptide sequences and fragments that can also be adapted for use as disclosed herein.

In addition, it should be understood that the techniques and/or sequences disclosed herein can be combined with each other, in various ways that will be apparent to those skilled in the art. As just one example, a gene construct can be developed, for expressing a gene encoding for a mature neurotrophin in BBB-straddling neurons, which encodes a polypeptide that contains both: (i) a pre-pro-BDNF leader sequence, at the N-terminus of the sequence encoding the mature neurotrophin, and (ii) an "epitopic tag" placed elsewhere in the sequence encoding the mature neurotrophin. Similarly, it should be recognized that other, additional genetic engineering techniques or sequences that are now known or hereafter discovered may also be adapted for use as disclosed herein.

As one example of a technique for increasing the expression of a passenger polypeptide carried by a genetic vector, the polypeptide coding sequence can be placed under the control of a powerful "gene promoter" sequence that will drive high levels of transcription of mRNA strands containing the coding sequence. Various promoter sequences that act as strong promoters in human cells (including human neurons) are known, and include, for example, promoter sequences derived from various types of pathogenic viruses, such as a cytomegalovirus (CMV) promoter, a Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter, and a simian virus 40 (SV-40) "early" promoter.

Alternately, in some cases, it may be preferable to place one or more genes (such as, for example, a gene which encodes a marker polypeptide) under the control of a so-called "inducible" promoter, which will be active only under certain conditions or when a certain compound is present.

Also of interest herein is a class of gene promoters usually referred to as "tissue-specific" promoters. These types of promoters cause a polypeptide coding sequence to be efficiently expressed into mRNA, only in specific types of tissues or cells. If a gene construct containing a tissue-specific promoter is delivered into the wrong cell or tissue type, those "non-intended" cells usually will not have the transcription factors or enzymes which can recognize that particular tissue-specific promoter; therefore, the gene construct will not be expressed (or will be expressed only at low rates) in those types of "non-intended" cells. Accordingly, tissue-specific promoters offer potentially very useful candidates for evaluation as described herein.

From published transgenic animal studies, a number of tissue-specific promoters have already been identified and published, and their number is increasing as additional studies on transgenic animals are published. The following non-exhaustive, non-exclusive list contains several examples of tissue-specific promoters that may be useful in this invention:

(i) tissue-specific promoters that appear to be much more active in olfactory receptor neurons than in other classes of neurons, include an olfactory marker protein promoter described in Servenius et al 1994, and an M4 olfactory receptor protein promoter described in Qasba and Reed 1998;

(ii) a tissue-specific promoter that appears to be much more active in nocioceptive sensory neurons than in sympathetic neurons, as described in Watson et al 1995. This gene, which expresses "minimal calcitonin gene related peptide" (CGRP), may also offer a

chemically-inducible gene promoter as well, since it appears to be much more active in the presence of nerve growth factor (NGF). Therefore, if the CGRP gene promoter is used, gene expression in sympathetic neurons could be minimized, which can help minimize undesired side effects. This type of gene promoter may be especially useful, since nocioceptive sensory neurons as well as sympathetic neurons are known to internalize NGF, via receptor-mediated endocytosis. Since both nocioceptive and sympathetic neuronal types will likely be transfected, if peripheral administration is used with a non-viral vector that exploits NGF-receptor-mediated endocytosis to target gene delivery to accessible neurons, the ability to create a gene construct that will not be expressed at substantial levels inside transfected sympathetic neurons, by using a gene promoter such as the CGRP promoter, may be highly advantageous.

(iii) a tissue-specific promoter which appears to be much more active in spinal motor neurons than in nocioceptive or other sensory neurons, and which drives expression of the alpha-1 subunit of glycine receptors, as described in articles such as Bechade et al 1994, Rajendra et al 1997, and Zafra et al 1997.

In general, where a transgene construct has been shown to restrict expression of a selected gene to a particular class of neurons in a line of transgenic animals, the same pattern of restricted gene expression can be expected when an appropriately prepared gene construct (containing the same promoter and associated gene expression elements) is delivered *in vivo* by viral or non-viral vectors. Thus, reference should be made to transgenic animal protocols (e.g., Causing and Miller 2000) when designing gene expression constructs.

In a related and similar manner, it also may be possible and desirable to use naturally-occurring or synthetic promoters and/or enhancers to further stimulate, restrict, or control expression of a foreign gene in various ways. For example, it may be possible to establish or increase foreign gene expression in injured neurons, without substantially affecting other neurons, by using a gene promoter which appears to be specifically activated after a nerve lesion, but which appears to be otherwise silent (see Funakoshi et al 1998). Alternately, a glucocorticoid-responsive promoter may be used to drive gene expression after neurological insult when, as a result of the stress response, glucocorticoid-adrenal stress steroids are present in high levels (e.g., Ozawa et al 2000). Alternately, the latency-associated transcript promoter from herpes simplex viruses (Lachmann et al 1997) is likely to drive gene expression in a manner that may be more prolonged than can be achieved by

various other promoters.

As another example of a technique that can be used to increase protein expression, scientists who create engineered genes for transfection often delete one or more "non-preferred" codons and replace them with "preferred" codons. The distinction between preferred versus non-preferred codons arises from the fact that most species (and many different cell types, within a certain species) have evolved with a repertoire of both: (i) preferred codons, which will pass quickly and without delay through the translation system inside ribosomes, and (ii) non-preferred codons that will slow things down, inside ribosomes. This system of preferred and non-preferred codons provides a cellular mechanism that is highly useful for regulating gene expression, so that properly balanced quantities of thousands of different proteins can all be present at suitable concentrations, inside a single cell. However, in genetic engineering, by getting rid of non-preferred codons and replacing them with preferred codons, a genetic vector can bypass the normal control system, and drive the production of unusually high levels of a foreign protein. This type of approach is described in numerous published works, such as US patent 5,795,737 (Seed et al, 1998) and various articles cited therein.

Another known genetic engineering trick involves "cysteine-depleted" variants of a polypeptide. As is well known, residues of cysteine (an amino acid with a highly reactive sulfhydryl group, --SH) in a polypeptide chain tend to react with other cysteine residues. When two cysteine residues react with each other, they form a disulfide bond. Disulfide bonds are very important in ensuring that, when a polypeptide is being synthesized inside a cell and is being subjected to "post-translation processing", it will be folded into its proper three-dimensional configuration. However, when genetic engineering is used to create proteins, cysteine residues that are not involved in disulfide bonds may generate problems and impede the desired result. Such problems often can be avoided and overcome by replacing one or more cysteine residues, at certain locations in a polypeptide sequence, with other residues. This is done by replacing the codon which specifies a cysteine residue, at some particular location in a gene, with a different codon that will specify some other non-cysteine amino acid residue. These types of cysteine-depleted "muteins" are described in various US patents, such as 4,737,462.

Genetic engineering techniques also can be used to add a so-called "leader sequence" and/or "signal sequence" to a foreign polypeptide that is encoded by a genetic vector as disclosed herein. Although the term "leader sequence" is not always used consistently or

precisely, it generally refers to a polypeptide sequence which has either or both of the following traits: (i) it causes or promotes the transport of the resulting leader-plus-polypeptide molecule to a certain location in a cell, or the secretion of the leader-plus-polypeptide by host cells, as can be demonstrated when the leader sequence is added to foreign polypeptides that normally do not undergo that type of transport or secretion; and/or, (ii) it is the portion of an initial polypeptide which is subsequently cleaved off, by natural "post-translational processing," from a smaller version of the final (mature) polypeptide. In nearly all cases, a leader sequence will be positioned at the N-terminus of a polypeptide; that is the end (often called the "head") which is created first, and which emerges first from the ribosome as the polypeptide is created.

Similarly, the term "signal sequence" is not always used consistently or precisely. In general, it refers to a polypeptide sequence that leads to some particular type of result, effect, or process, as can be demonstrated by the ability of that signal sequence to impart the same result or process to other peptide sequences that normally do not undergo that result or process. As examples, signal sequences can lead to (i) enclosure, sequestering, or other processing or "packaging" of a polypeptide into vesicles or other compartments; (ii) transport of a polypeptide to a particular component or region of a cell; or (iii) secretion of a polypeptide by the host cell. Accordingly, "signal sequence" is used more broadly than "leader sequence", and generally includes leader sequences. In addition, there is no implication that a signal sequence must be cleaved off of an initial polypeptide, to create a mature final version of the polypeptide; signal sequences are often retained by fully mature polypeptides.

Various neuronal leader and/or signal sequences are known that are believed to increase either or both of two processes that are highly useful in this invention. Those two processes are: (i) anterograde transport of a polypeptide, out of the main body of a neuron and through a "central projection" which will transport the polypeptide, while still inside the cell where it was synthesized, across the blood-brain barrier and/or closer to a desired targeted area of the brain; and/or, (ii) secretion of a polypeptide, by a neuron, at synaptic or other terminals belonging to the neuron.

One such type of leader sequence which may be highly useful, in various settings, is a leader sequence that is initially found in a polypeptide called "pre-pro-BDNF", where BDNF is the acronym for brain-derived neurotrophic factor. BDNF is a homologue of nerve growth factor (NGF), but unlike NGF, pre-pro-BDNF is known to be efficiently

transported, in an anterograde direction, by nocioceptive neurons. This has been confirmed by animal tests, showing that BDNF that was expressed inside nocioceptive neurons can be detected later in spinal tissue. The shorter, final (or "mature") form of BDNF is created when the leader sequence is cleaved off of the initial longer polypeptide. The BDNF polypeptide, and the pre-pro-BDNF leader sequence, are described in a number of published articles, including Conner et al 1998, Altar et al 1999, and Tonra 1999. Anyone skilled in this particular art who evaluates what is known about the BDNF polypeptide and the pre-pro-BDNF leader sequence, will recognize that this leader sequence, and any other peptide leader sequence which is known to enable or increase anterograde transport within neurons and/or secretion by neurons, may be highly useful in increasing the success rates and efficacy levels of this invention.

EPITOPIC TAG SEQUENCES

Another genetic engineering technique also deserves attention, since it may be highly useful in this invention. This technique involves "epitope" sequences, tags, and constructs.

The prefix "epi-" refers to an exposed and accessible surface; as examples, the epidermis is the outermost dermal (skin) surface, and the epithelium is the exposed surface of a mucous membrane.

Similarly, the term "epitope" is used to refer to a surface-exposed domain of a protein which can be firmly bound by an antibody. An antibody does not wrap itself around the entire surface of an antigenic protein; instead, a localized binding domain which is part of the antibody will bind to a localized binding domain which is part of the antigen, in a manner that is analogous to two jigsaw pieces fitting together along one (and only one) edge of each piece.

By using tests in which digested (cleaved) fragments of an antigenic protein are passed through a column in which monoclonal antibodies have been affixed to tiny beads, it is not difficult to identify the epitopic region of an antigenic protein, with respect to a particular line of monoclonal antibodies. However, different lines of monoclonal antibodies will bind to different surface areas of an antigenic protein; accordingly, a protein generally has a number of different epitopic regions, and the common factor that determines which regions are epitopic is whether a region is exposed, and accessible to antibodies, on the surface of the protein. For this reason, epitopic analysis is often used by researchers to help them determine the three-dimensional structure of a complex protein; as a general rule, the

amino acid sequences which show up as epitopic sites in a protein are the amino acid sequences that are exposed, and accessible to antibodies, on the surface of the protein.

The practice of using epitopic "tag" sequences in genetic engineering arises from the following fact: it is relatively easy to create and identify monoclonal antibodies that will bind, with very high levels of selectivity, to known epitopic sequences. Several such monoclonal antibody lines (and the corresponding high-affinity antigenic amino acid sequences to which they bind) are well-known; examples include monoclonal antibody lines that will bind to known amino acid sequences derived from polypeptides such as c-myc, and a hemagglutinin protein from the influenza virus.

Because polypeptide expression in cells starts at the N (amino) terminus, and ends at the C (carboxy) terminus, there is a generally high probability that amino acid segments located at or near the C terminus will be exposed on the surface of the protein. This arises from the fact that the folding and shaping process which generates the final three-dimensional polypeptide will begin taking place as the strand is being created and extended, since various amino acid residues or domains located along the strand in specific locations will begin attracting or repelling each other as the strand is being formed. Although this depiction is highly simplified and does not adequately address the subtleties of protein folding, one can envision the first part of the strand that emerges from a ribosome as forming the "core" of the emerging polypeptide, and the rest of the strand generally being wrapped around or otherwise added to the initial core.

Because of this factor, amino acid sequences that are at or near the "tail end" (the carboxy terminus) of a polypeptide have a relatively high likelihood of being exposed and accessible, as epitopic sites, on the surface of the polypeptide. To take advantage of this factor, and to reduce the risk that insertion of an epitopic site into a central domain might disrupt or degrade an essential activity or trait of the polypeptide, the normal practice is to position epitopic tag sequences at or near the tail end (the carboxy terminus) of a polypeptide that is being modified to include an epitopic tag. This generally offers the best approach to initial research using epitopic tags, and experimental data obtained using this approach can be used to optimize subsequent efforts, if necessary.

Alternately or additionally, if the full three-dimensional structure of a polypeptide molecule is known, and if the polypeptide is known to have an "active" site that is crucial for catalytic activity or receptor binding, it may be possible to insert a surface-accessible epitopic site into the polypeptide, on the opposite side of the molecule.

By using relatively simple *in vitro* screening tests, any such modified "tagged" protein can be tested to determine whether it still has the desired activity, and tagged proteins which pass those initial tests can be tested more extensively using *in vivo* animal models.

Accordingly, the genetic vector system disclosed herein allows a gene construct to be created, in which a unique or at least highly uncommon and detectable "epitopic tag sequence" can be added to (or inserted into) nearly any type of known protein (such as, for example, nerve growth factor). Using known genetic engineering techniques, it is a straightforward procedure to modify the coding sequence of a gene construct carried by a genetic vector, in a manner which will add a relatively small number of additional codons to the "native" coding sequence, or which will substitute and replace a few codons in the native coding sequence by other codons that do not normally appear in the native protein.

In various forms of research and treatment that are taught herein, genetic vectors carrying gene constructs that will express epitope-tagged polypeptides can be highly useful, since they can make it much easier (and in some cases, they may be required to make it possible) for researchers and physicians to monitor and quantify the results and effects of the genetic treatments disclosed herein. As one example of the types of problems that can be encountered when naturally-occurring polypeptides must be analyzed, under the current state of the art, naturally-occurring nerve growth factor (NGF) tends to be degraded by most types of tissue fixation methods; in addition, NGF is very difficult for even highly skilled researchers to measure accurately, and high degrees of homology and antibody cross-reactivity exist, in the different versions of NGF that are found in animals as widely different as rats and humans. Therefore, epitope-tagged versions of NGF (and of various other neuroactive polypeptides) can be used to avoid or at least minimize such difficulties.

It also should be noted that epitope-tagged polypeptides are used much more frequently and widely in research, than in clinical practice on humans. In general, epitope-tagged polypeptides are used most commonly for developing, optimizing, and proving the effects and efficacy of a certain treatment approach, in cell culture tests and animal tests. In addition, they sometimes are used in small-scale "Phase 1" or "Phase 2" human clinical trials, which are authorized under "Investigational New Drug" applications in the U.S., and under similar governmental review and approval mechanisms elsewhere. By the time a treatment is ready to be tested in larger multi-site Phase 3 clinical trials, any epitopic or other "foreign" sequences usually will be removed, to reduce the risk of generating an

immune response involving antibodies that would recognize and attack the epitopic sequence.

IN VIVO IDENTIFICATION OF ENDOCYTOTIC LIGANDS, FROM PHAGE LIBRARIES AND COMBINATORIAL CHEMISTRY

As mentioned in the Summary section, a new type of in vivo screening method has been created by the Applicants, for identifying and isolating endocytotic ligands that will be taken into, and transported within, neuronal fibers. Since this new method offers a preferred mode of carrying out this invention, it is disclosed herein in detail, to enable those skilled in the art to use this method to create improved genetic vectors that can be used to delivery polypeptides into BBB-protected CNS tissue.

To fully understand the *in vivo* screening method that is involved, and the endocytotic ligands that can will result from this screening method, some background information needs to be provided on endocytotic ligand receptor proteins. Like nearly all cell surface receptors, endocytotic receptors straddle the outer membrane of a cell. Accordingly, they have three distinct domains: (i) an extracellular portion, which is exposed and accessible to molecules carried by the aqueous fluids that surround and contact the cell; (ii) an interior portion which is embedded in the lipid bilayer membrane that encloses the cell; and, (iii) an intracellular portion, which is exposed to the aqueous cytoplasm inside the cell.

In general, ligand receptors have two traits that distinguish them from various other types of non-ligand receptors. The first trait is that each particular type of endocytotic ligand receptor will interact with, and will be triggered and activated by, only a single type of extracellular molecule (or, in some cases, only a very small and limited group of structurally similar extracellular molecules). This binding mechanism can be regarded as being directly comparable to a "key-in-lock" arrangement; only a small number of keys, having certain exact and limited sizes and shapes, can fit into a certain lock. This trait distinguishes ligand receptors from, for example, various types of transport proteins that will grab nutrients floating outside the cell, and transport those nutrients into a cell interior.

The second distinguishing trait is that, when a binding reaction does occur between a ligand and an endocytotic ligand receptor protein, the two molecules will remain bound to each other for an extended period of time. This distinguishes ligand receptors from neurotransmitter receptors that are triggered by acetylcholine, glutamate, and other classical

neurotransmitters. Instead of being measured in milliseconds (as occurs with most neurotransmitter receptors), ligand binding reactions usually can be measured in minutes, hours, or even days.

In an "endocytotic" ligand binding reaction, the ligand binds to the ligand receptor in a way that forms a ligand-receptor complex, which will then be taken inside the cell, where both the ligand and the receptor typically are eventually digested, so that their building blocks can be recycled. To understand how and why this occurs, one should consider polypeptide hormones such as growth hormones, nerve growth factors, etc., that can cause permanent alterations in the size, health, nerve or reproductive system, or other traits or organs of an animal. If these types of hormones were released by their receptors, they would float back into the extracellular fluid, and they could then contact and activate additional receptors on other cells, in a manner that could lead to unregulated and potentially uncontrollable effects.

As used herein, the terms *endocytosis* and *internalization* are used interchangeably, to refer to a cell-driven process in which an extra-cellular molecule (other than a nutrient or oxygen) which has become bound in a specific manner (usually referred to as "affinity binding") to a molecule on the cell surface, is drawn into the cell interior. This process includes receptor-mediated endocytosis, involving ligands that bind to receptor proteins. It also includes the process in which ligands that bind specifically to other types of cell surface molecules (including surface carbohydrates) form other types of ligand complexes that are drawn into a cell. The terms endocytosis and internalization also include the processes called *pinocytosis* (which involves the intake of very small particles, or soluble molecules) and *phagocytosis* (which involves the intake of larger particles, such a virus particles or bacterial cells), provided that such processes involve specific binding of a ligand molecule to a cell surface molecule, in a manner that forms a complex which is subsequently internalized by the cell.

The processes ino\volved in endocytosis are well-known, and are discussed in numerous reference works, such as Alberts et al, *Molecular Biology of the Cell* (third edition, 1994), pages 618-626 (which describe and illustrate endocytosis) and pages 636-641 (which describe clathrin proteins and triskelions, which aid and facilitate the process of endocytosis), and pages 731-734 (which describe and depict internal conformation changes that occur when a ligand binds to a membrane-straddling protein).

In most types of animal cells, endocytosis of ligand-receptor complexes involves

transport of the lipid vesicle only over very short distances, since the typical diameter of most animal cells is in the range of about 10 microns, or 1/100 of a millimeter.

However, ligand-receptor complexes in neurons can travel much longer distances. Many neurons in the brain and spinal cord have long fibers (including axons, dendrites, and "processes") that extend for multiple centimeters, and some types of neurons that carry nerve signals in humans from a hand or a foot to the spinal cord (or vice-verse) have fibers that extend more than a meter. Accordingly, endocytosis that occurs within these types of long fibers must be able to carry a ligand-receptor complex all the way from the most distant tip of the neuronal fiber, where some ligand-receptor complexes will first enter a neuron, to the main cell body of the neuron, regardless of how far that distance may be.

As mentioned previously, "retrograde" transport occurs when a molecule is transported from an extremity (or "terminal") of a neuron, along an axon or dendrite, toward the main body of the cell, where the nucleus is located. Transport in the opposite direction is called "anterograde" transport, and the term "axonal transport" should also be recognized and understood. Many types of nerve cells have a single main fiber, which is the largest fiber that extends out from the main cell body. That largest fiber is usually called the axon. The term "axonal transport" includes and refers to any form of transport of molecules within an axon, regardless of which direction the molecules are travelling (i.e., toward the cell body, or away from the cell body). Therefore, retrograde transport that occurs within an axon is a form of axonal transport. However, the term "retrograde transport" is preferred herein, since it also indicates the direction of travel.

Not all candidate ligands that can bind to an endocytotic ligand receptor can activate and drive the internalisation process to full completion. As an example, monoclonal antibodies can be generated, rather easily, that will indeed bind to known endocytotic receptors, but there are few reports of such antibody preparations that can successfully trigger and then complete the entire process of receptor-mediated endocytosis. One example of an antibody preparation that reportedly can trigger and then complete the process of receptor-mediated endocytosis in rat neurons is a monoclonal antibody, initially designated as IgG-192 and subsequently called MC192, described in Chandler and Shooter 1984. The MC192 antibody binds specifically to a rat neuronal receptor that was known in the mid-1980's as the "low affinity nerve growth factor receptor", and that was subsequently designated as the p75 receptor. Years after the MC192 monoclonal antibody was created, other researchers reported that when it was radiolabelled and injected into rats, it was

internalised and retrogradely transported into the cell bodies of neurons that express the p75 receptor on their surfaces (Yan et al 1988).

That example may prove that it is theoretically possible to develop an antibody that can trigger and then complete the process of ligand-receptor endocytosis; however, major obstacles still remain before that type of research discovery can be used in human medicine, and two crucial sets of questions immediately arise.

The first set of questions center on the difficulties of extending those types of findings, to human medicine. Obviously, it is highly problematic and in many cases illegal to inject antigens or unproven antibody fragments into humans. Even more importantly, the screening tests that would need to be performed, in order to prove that some particular antibody type that works well in animal tests can also work well in humans, would be very difficult and potentially impossible, unless they are done in ways that currently are not acceptable in human research. If one begins to seriously contemplate the obstacles that would confront such research tests (which are likely to require samples of spinal tissue to be removed and then analyzed to determined whether radioactively-labelled tracer molecules actually reached the spinal cord), one ends up pondering tests on murderers who are condemned to be executed within the next few days, or on people who are going to die of cancer or other terminal diseases within the next few days. However, tests involving removal of solid tissue from a human spinal cord, so the tissue can be analyzed, are not allowed in any industrial nation where modern medical practices are used.

The second set of difficult questions centers on the issue of what types of molecules an "endocytotic receptor antibody fragment" might be able to carry along with it, into a cell interior, if the antibody fragment itself can work as hoped as an endocytotic trigger. Clearly, there is no therapeutic value in having antibody fragments, with nothing else attached, pulled into the interiors of neurons. Instead, such antibody fragments must be regarded merely as vehicles (or as locomotives, which could be used to pull a train). They will not be useful unless they can carry or pull some type of "passenger" or "payload" molecule into the neurons they are entering.

However, it must be recognized from the outset that coupling any additional molecular fragment to an endocytotic antibody fragment will necessary enlarge the resulting conjugate. Depending on how much larger the conjugate will be, this enlargement may substantially reduce the ability of the conjugate to be pulled into neurons with the same level of efficacy as the antibody fragments alone. There is no good way to answer that type

of question at an early stage, during the research that will be necessary on any such antibody fragment. Instead, the transport vehicle must be evaluated and proven to work, on its own, before it becomes worthwhile to test that vehicle's ability to carry (or pull) passenger or payload components.

Accordingly, since the reports published to date indicate that only a small subset of the antibodies generated against endocytotic receptors may be capable of mimicking a natural ligand's ability to trigger and then complete the process of receptor-mediated endocytosis, the process of testing each of dozens or even hundreds of monoclonal antibody candidates, to identify rarely-occurring internalising antibodies, renders those problems even more difficult and expensive.

It should also be noted that *in vitro* screening processes, to evaluate candidate endocytotic ligands by using cell culture conditions, suffers from two major problems. Those two problems have effectively limited progress in that field to work on cancer cells, and on blood cells that are involved in autoimmune diseases.

To understand these two problems, one must first note that most evaluations of "libraries" or "repertoires" of candidate ligands, in an effort to identify and isolate the rare "needle-in-a-haystack" ligands that can activate and drive endocytosis, will usually begin with either: (i) a phage display library, or (ii) a preparation that was created by "combinatorial chemistry". Phages (which are viruses that can infect bacterial cells) and phage display libraries are described in articles such as Koivunen et al 1999, Cabilly 1999, Shusta et al 1999, Larocca et al 1999, 2001, and 2002, Rader 2001, and Manoutcharian et al 2002. Combinatorial chemistry is described in articles such as Lockhoff et al 2002, Flynn et al 2002, Edwards et al 2002, Ramstrom et al 2002, Ley et al 2002, Lepre et al 2002, Liu et al 2003, Edwards 2003, and Geysen et al 2003.

When researchers try to use *in vitro* cell culture preparations, in their efforts to screen phage display libraries to identify endocytotic polypeptide ligands, they encounter two main problems: (i) multiple different phages will usually bind to multiple different proteins and other molecules, on the surfaces of cells, without being taken into the cells; and, (ii) it is very difficult to rinse off, wash off, or otherwise reliably remove any and all phages that are clinging to the surfaces of cells, and that have not been taken inside the cells, without killing and lysing the cells or otherwise creating severe problems that will interfere with other desired processing of the cells and/or internalized phages. Because of these two factors, it is very difficult to prevent "false positives" from being selected.

Accordingly, *in vitro* screening of phage libraries for endocytotic ligands has succeeded to a significant degree only in working with cancer cells or blood cells, which can be grown readily in liquid cell culture solutions. By contrast, *in vitro* screening of phage libraries for endocytotic ligands has not succeeded, to any substantial degree, in cell cultures that involve anchorage-dependent cells of the type that generate cohesive tissues.

The problems summarized above apply to even the simplest tissue culture systems, where all of the cells can be clonal duplicates and have exactly the same receptor types. The notion of attempting to carry out phage library screening tests in an intact and still-living animal (where multiple different tissue and cell types, each with their own specialized set of receptors and other surface molecules, must coexist in close contact with each other, and with blood and lymph constantly circulating through and between the different tissues regions and cell types) simply is not within the mindset of ordinary artisans who are skilled and practiced in the art of phage library screenings, and who understand the considerable difficulties of doing it successfully even in the simplest cell culture conditions.

Accordingly, there have been many efforts, and much progress, in using phage display libraries to develop improved genetic engineering methods and vectors that can be used to genetically transform and treat cancers, and autoimmune diseases. However, there have been few efforts, and only very paltry and limited progress, in using phage display libraries to treat other diseases, or to create genetic vectors that can enable the transformation of neurons and other cells that are present in cohesive tissue, inside the body. Under the prior art, the challenges and difficulties of eliminating false positives, when phage display libraries are screened for endocytotic uptake into neurons and other cohesive tissue cells in intact animals, in *in vivo* tests, have been so severe, and so formidable, that they have effectively blocked and prevented any substantial progress in that field of research. Prior to this invention, no one had figured out how to make practical use of phage display libraries, to accomplish the results that can now be achieved by the *in vivo* screening method of this invention.

Accordingly, this newly developed *in vivo* screening method can be described as follows, with reference to Figures 7-9. Additional information is contained in Examples 22 through 31, below.

In a preferred embodiment, this *in vivo* screening process can use the sciatic nerves of rodents, such as rats or mice. Both of these species are inexpensive and easy to breed and raise, and they have become the standard animal models used in most genetic research

in small mammals. A huge foundation of information, species-specific biomolecules (including gene promoter sequences, gene coding sequences, monoclonal antibodies, etc.) and specialized animal strains, have been developed for genetic work with mice, and gateways that can be used to access that information are freely available on websites such as www.informatics.jax.org and www.ncbi.nlm.nih.gov/genome/seq/MmHome.html. Although the corresponding information, reagents, and strains for rat genetics are somewhat less, they are still enormous and quite useful, and can be accessed through websites such as http://rgd.mcw.edu, http://ratmap.gen.gu.se, and www.hgsc.bcm.tmc.edu/projects/rat.

Because of the larger size of rats, it is easier to work with their sciatic nerves (which pass, on each side of the animal, from the spinal cord, through one hip and leg, down to the foot) than with mice. This can be done by known methods, such as discussed below.

However, even in mice, the sciatic nerves are long enough and sufficiently distinct to enable the required surgical manipulations, using the procedures disclosed herein (especially if such manipulations are carried out by researchers who have done such work before). In addition, it should be kept in mind that surgical manipulations in mice can be done with the aid of binocular microscopes, and surgical tools that are commonly used by ophthalmologic surgeons and neurosurgeons. Additional comments on surgical methods are contained in Example 25, below.

It should also be noted that other types of laboratory animals (which may include primates, non-mammalian vertebrates, or even some types of invertebrates) can also be evaluated for potential use in this type of *in vivo* screening, if desired. In particular, some animals are known to have exceptionally large neuronal axons; as one example, some types of squids have a "giant axon" that controls the muscles that drive propulsion. In the same way that Chinese hamster ovary (CHO) cells became widely used in research laboratories because they contain unusually large cell components, squids or other animals that have unusually large nerve fibers or bundles can be used as disclosed herein, if desired.

The placement of candidate ligands in contact with a sciatic nerve bundle can be done in a manner that is schematically illustrated in FIG. 7, and discussed in more detail in Examples 25 and 26, below. Briefly, if an inducible receptor (such as the low-affinity p75 nerve growth factor receptor) is going to be targeted, a first ligature 1102 is emplaced and then tightened around the sciatic nerve bundle 1090. This ligature 1102 is created by placing a strand of suture material around the nerve bundle, and then tightening the loop

and tying it off, in a manner that creates a constriction that acts as a tourniquet, by hindering the normal flow of fluids and molecules inside the nerve fiber.

As shown in FIG. 7, ligature 1102 can be placed adjacent to the "tibial branch bifurcation" 1092, where the sciatic nerve bundle 1090 divides into two major branches, which serve different parts of the leg and foot. Ligature 1102 preferably should be placed *above*, and fairly close to, the tibial branch bifurcation 1092. As mentioned in the Background section, the terms "above" and "proximal" indicate a location closer to the animal's spinal cord (toward the right side of the drawing shown in FIG. 7). By contrast, the terms *below* and *distal* indicate a location farther away from the spinal cord, and closer to the leg or foot (toward the left side of the drawing in FIG. 7).

The purpose of ligature 1102 is to increase the number of p75 receptors that will be expressed on the surfaces of the sciatic nerve bundle. The p75 receptor interacts with certain neurotrophic factors (also called nerve growth factors) which are polypeptides that have hormone-like effects on nerve cells. The best known such molecule was called nerve growth factor, since it was discovered fairly early in the process; as additional such molecules were discovered, they were given names such as brain-derived neurotrophic factor (BDNF), neurotrophin-3, and neurotrophin-4/5. The neurotrophins effectively stimulate neurons in ways that generally lead to increased metabolic activities, the formation of additional synaptic connections with other neurons, etc. If a neuronal fiber of a motor neuron is injured or distressed, one of the ways the motor neuron responds is by increasing the number of p75 receptors on its neuronal fiber, which may give it a better chance to grab and bind any nerve growth factor molecules that happen to be in the surrounding extracellular liquids. This process of "upregulating" certain types of neuronal receptors on the surfaces of nerve fibers has been discovered and shown to occur in certain neurodegenerative diseases, notably including amyotrophic lateral sclerosis, also called ALS, Lou Gehrig's disease, and motor neuron disease. It also occurs after various types of trauma.

Accordingly, ligature 1102 is designed to exploit that type of neuronal response. By emplacing and tightening a loop of suture material around the nerve fiber, in a manner which creates a tourniquet that blocks the flow of fluids through the fiber, it is possible to increase the number of p75 receptors along the length of the nerve fiber, between the spinal cord and the ligature site. Based on various tests, including staining tests that use monoclonal antibodies that bind to p75 receptors, the increase in p75 receptors is estimated

to be about 10 to 15-fold.

This receptor expression response, by a nerve fiber, to a constrictive ligature, occurs over a span of roughly a week. Therefore, after ligature 1102 is placed and tightened around the sciatic nerve bundle, the wound should be closed and sutured, and the animal should be allowed to recover, for at least several days and preferably for about a week, before the next surgical procedure is performed.

During the second procedure, two different sites will be surgically opened, roughly 2 to 3 centimeters apart from each other. One site will be close to the same site where the ligature 1102 was placed; indeed, the surgical opening can be located at the same site as before. At this site, the sciatic nerve bundle is cut (i.e., transected), by using a scalpel or scissors, in a manner that generates two ends (which can be blunt, angled, etc.). The cuts are made just above and below ligature 1102, and a small portion of the nerve bundle which contains the ligature can be excised and discarded. These cuts will create two ends of the nerve bundle, designated as distal end 1094 (which will no longer be active), and proximal end 1130 (on the side that is toward the spinal cord).

At the site where the sciatic nerve bundle 1090 is cut, a bolus of material 1150 is emplaced. This bolus 1150 is made a porous and permeable material (such as a collagen gel foam) that contains a large number of phage particles (preferably in the millions or billions of "colony forming units" (cfu)). This bolus 1150 should be emplaced and secured at this site, in a manner that will promote sustained intimate contact between (i) the phage particles that are contained in bolus 1150, and (ii) the cut end 1130 of the sciatic nerve bundle 190. This type of emplacing and securing can be done by means such as:

(a) using a strand of suture material 1132 to tie together the two cut ends 1094 (i.e., the distal cut end) and 1130 (i.e., the proximal cut end) of the sciatic nerve bundle 1090; and,

(b) wrapping and securing a small sleeve or cuff 1136, made of a watertight material such as silicone rubber, around bolus 1150 and around the two ends 1094 and 1130 of the sciatic nerve 1090, in a manner which encloses the bolus and the two nerve ends inside a small watertight cylindrical volume. If desired, the sleeve 1136 can be secured by wrapping and tying one or more suture strands around it, by placing a droplet or bead of adhesive material on the outer surface of the sleeve, or by any other suitable means.

Accordingly, this site, where the bolus of material containing phage particles is emplaced, can be referred to as either the phage placement site, or the phage contact site.

This is the location where the library or repertoire of phage particles will contact the cut end of the sciatic nerve. Phage particles that happen to display, on their surfaces, polypeptide sequences that will trigger endocytosis (such as through a p75 receptor on the surface of a nerve fiber) can be internalized by the nerve fibers, at this site.

At a separate and distinct site, preferably located roughly a centimeter or more away from the phage placement site, a second site is surgically opened, and a second ligature 1202 (formed by a loop of suture material) is emplaced and then tightened and tied around the sciatic nerve bundle. Since the rat hip offers a convenient location, far enough away from the phage placement site to eliminate any significant risk of false positives caused by phages clinging to the outsides of sciatic nerve fibers, this site preferably should be in the hip region, and it is referred to herein as the hip ligature site. The hip ligature 1202 will act as a constriction or tourniquet around the sciatic nerve bundle 1090, and must be tight enough to substantially hinder the travel of fluids or molecules, inside the nerve fibers, across that blockage point. Accordingly, this constriction will generate a phage accumulation zone 1204, inside the nerve bundle and distal to the hip ligature 1202.

The rat wounds are closed and sutured, and a suitable span of time (such as about 18 hours) is allowed to pass, to give phage particles that happen to be carrying ligands that can effectively activate and drive the process of endocytosis, enough time to enter the nerve fibers, and then be retrogradely transported through a significant length of the nerve fibers, toward the spinal cord).

After that span of time has passed, the rat is painlessly sacrificed, the site of the hip ligature is opened, and a segment of the sciatic nerve bundle immediately adjacent and distal to the hip ligature is removed (harvested). This short bundle of nerve fibers is then divided into small pieces, and processed using chemicals that will partially digest cell membranes (which are made of lipid bilayers) without damaging the phage particles. This processing allows the collection and isolation of viable phage particles that had been internalised into the nerve fibers.

The phage particles that are selected by a round of *in vivo* screening as disclosed above can be reproduced and/or manipulated in any way desired. As examples, any and all of the following procedures can be carried out, using phage populations selected by the *in vivo* screening process disclosed herein:

(1) if the phages are "phagemids" (which merely requires the phage to contain a bacterial origin of replication), they can be amplified (reproduced), by using *E. coli* cells

without helper phages, in ways that will generate double-stranded DNA in plasmid form. It should be noted that nearly all modern phage display libraries use phagemids, since they enable various useful procedures, including the synthesis of circular plasmid DNA in any desired quantity. All phage display libraries used herein were phagemid libraries.

(2) by using *E. coli* cells plus helper phages, the selected phages can be amplified in ways that generate new and fully infective phage particles containing ssDNA. These phage particles can be used as the starting reagents in another cycle of *in vivo* screening, which (during the early cycles) can be used to refine an "enriched" population of endocytotic phages into an "elite" population that is likely to contain ligands that are even more effective at triggering and driving endocytosis.

(3) when enough selection cycles have been completed to suggest that a suitable point for phage analysis has been reached (in most cases, this is likely to happen after at least one, up to about three cycles of screening, or possibly more in some cases), the phages selected by the last round of *in vivo* screening (or, indeed, by any round of *in vivo* screening) can be used to create either or both of the following: (i) any desired quantity of double-stranded or single-stranded DNA, for nucleotide sequencing to determine the exact sequence of the gene that encoded a particular endocytotic ligand; and/or, (ii) any desired quantity of the coat protein which carries an endocytotic ligand, in a soluble form that can be processed and sequenced, to determine the amino acid sequence of the ligand domain in that particular coat protein.

Photographic Confirmation of In Vivo Screening Results

The efficacy and success of the in vivo screening process disclosed herein is depicted, visually, by the photograph in FIG. 8. This photograph was created during an actual test of this *in vivo* selection process, using fluorescent reagents to indicate the locations and concentrations of phages that were internalised within the sciatic nerve bundle (the phage preparation and staining reagents that were used in this test are described in Example 27, below). The left side of the photograph in FIG. 8 shows fairly high concentrations of fluorescent-labelled phages, in the nerve portion that corresponds to phage accumulation zone 1204 as shown in FIG. 7. The choked and narrow zone in the center of the photograph was created by the hip ligature 1202. The right side of the photograph shows the sciatic nerve on the proximal side of the hip ligature (i.e., in the direction of the spinal cord). Since very few or no phages were able to squeeze past the hip ligature 1202

and reach that part of the sciatic nerve, it shows almost no fluorescent labelling.

Use of In Vivo Screening with Combinatorial Chemistry

The general approach disclosed herein, for in vivo screening and identification of endocytotic ligands, can be adapted for use with candidate ligand molecules created by "combinatorial" chemical synthesis. Over the past 20 years, this branch of chemical synthesis and screening has become a highly active field, and an April 2003 search of the National Library of Medicine database revealed more than 900 review articles on this field of research. Recent review articles include Lockhoff et al 2002, Flynn et al 2002, Edwards et al 2002, Ramstrom et al 2002, Ley et al 2002, Lepre et al 2002, Liu et al 2003, Edwards 2003, and Geysen et al 2003. The synthesis methods and approaches described in those review articles can be used to provide a wide range of highly diverse combinatorial libraries.

One of the essential traits of any such combinatorial library is that it must be adaptable to at least one or more types of screening tests. Otherwise, a mixture of thousands or millions of different candidates would be totally worthless, since no one would be able to tell which particular compounds, in the mixture of thousands or millions of candidates, would be useful for some particular purpose.

Therefore, any type of combinatorial library will be created in a manner that provides the candidate compounds with some type of "handle" that can be used to identify or manipulate the candidates (or that can identify or manipulate those particular compounds that were modified, isolated, or otherwise distinguished by a reaction or screening process) in one or more useful ways.

A fairly generous variety of these types of "handles" are known, and the variety of known approaches will enable at least one and usually more of these "handles" to be adapted to *in vivo* screening of combinatorial libraries, using nerve fiber manipulations as disclosed herein. As examples, well-known classes of "handle" approaches that can be used to process and control combinatorial chemistry repertoires can include any and all of the following:

1. microscopic beads, tubes, or other solid surfaces, usually made of a plastic, starch, or similar compound. These beads or other solid surfaces usually serve as a substrate or "anchor", and provide (on their surfaces) reactive groups that will become attachment points for chemical chains that will be added to those reactive groups. If

desired, these types of microscopic beads can be created with diameters that are well suited for phagocytotic intake by mammalian cells.

2. special reactive moieties that occur only once in each candidate compound in a combinatorial library. These unique reactive moieties can be used to enable attachments, chemical reactions, or other manipulations, that can be used at any stage during or after a screening process is carried out, to precipitate, condense, or otherwise gather, isolate, conjugate, label, or manipulate particular candidate compounds that were transported to a target location, or that became involved in a chemical or cellular reaction of interest, or that otherwise acted differently from the unsuccessful candidates, during a screening test.

3. non-toxic fluorescent "labels" or "tags" that will emit light at one wavelength, when excited by light having a different wavelength. This enables the use of equipment called "flow cytometers" (also called cell sorters, and similar terms), to segregate cells or particles that have fluorescent activity. In a typical flow cytometer with sorting capability, millions of cells or particles can be passed through a narrow tube, one at a time, at a known and controlled velocity. At one location in the pathway, each cell or particle passes through a light with an excitatory wavelength. Individual cells or particles that contain or are attached to a fluorescent label or tag will respond by emitting light at the different wavelength. This fluorescence, which occurs within nanoseconds, is detected by an optical sensor which is tuned to the fluorescent wavelength. When that optical sensor detects fluorescent light emitted by a certain cell or particle, it triggers a tiny jet of gas or liquid, at a location slightly downstream in the flow path of the cells or particles. That jet of gas or liquid is timed to coincide with the passage of the fluorescent cell or particle, through a junction in the pathway. If the jet of gas or liquid pushes a fluorescent cell or particle to one side, in the flow path, it will enter a separate collection tube, which will carry it to a collection vessel. In this way, a flow cytometer can process millions of cells or particles within a span of hours or even minutes, and it can isolate even a single individual fluorescent cell or particle, out of a population of millions.

4. other types of labels or tags, such as compounds that include radioactive isotopes, or specialized molecular structures that can be located and tracked by sophisticated analytical methods such as magnetic resonance imaging, Raman scattering, etc.

5. whenever an assortment of candidate ligands includes or involve polypeptides, phage display libraries offer exceptionally powerful, flexible, and adaptable "handle" systems for working with such polypeptides. If even a single phage particle is isolated

which carries a highly effective and potentially useful ligand polypeptide, then that single phage particle can be grown rapidly into an entire clonal colony, which can provide an unlimited supply of both the polypeptide, and the gene which encodes that polypeptide, using procedures as described herein or as otherwise known to those skilled in the art.

Indeed, the PhD-C7C phage display library offers an example of a combinatorial approach that has been adapted for use with polypeptides. In this library, essentially random segments of short polypeptides, seven amino acids long, were created by combinatorial chemistry. These randomly-created short polypeptide sequences were incorporated into phage particles, and those phage particles provide the "handles" which can be used to manipulate, reproduce, and screen the combinatorial assortment of polypeptides in the PhD-C7C library.

As mentioned above, any combinatorial library must necessarily be created in a manner that will render it susceptible to at least one type of system or mechanism that enables researchers to handle and manipulate the candidate compounds in the library. Otherwise, it would be useless to generate such libraries, if they could not be screened by effective and logical methods. Therefore, the range and variety of methods that have been developed over the past 20 years, for screening combinatorial libraries, have become quite sophisticated and powerful. Accordingly, the *in vivo* screening methods disclosed herein can be regarded as merely providing one more new (and potentially powerful, and useful) method for screening candidate compounds that have been created by combinatorial chemistry.

Genetic Engineering Methods to Extend In Vivo Screening to Other Receptors, Other Species, and Other Classes of Neurons

Those skilled in certain related arts will recognize various ways in which this invention, initially developed and tested using the motor neurons of the sciatic nerve in rats, can be expanded and extended in several particular directions that will be of interest to research, physicians, and others.

As one example, those skilled in neuroanatomy and neuronal tracing studies will recognize ways in which this invention can be expanded beyond sciatic motor neurons, to enable its use: (i) with sympathetic processes emanating from the superior cervical ganglion and sensory processes emanating from the trigeminal ganglion sensory nerves, following injection of phage libraries into the anterior eye chamber; (ii) with olfactory receptor

sensory neurons (harvesting olfactory bulb tissue), trigeminal ganglion sensory and superior cervical ganglion sympathetic nerves, following administration of test libraries into the nasal cavity; (iii) with retinal ganglion cell sensory neurons, following injection into the posterior chamber of the eye; and, (iv) with various neurons of the central nervous system, following injection into the lateral or other ventricles of the brain. By such means, ligands targeted at endocytotic receptors that are naturally expressed by these particular neuronal populations can be identified and isolated, for use in diagnosing and treating disorders that involve those particular classes of neurons.

Similarly, those skilled in genetic engineering and molecular biology will recognize ways in which this invention, initially developed and tested using p75 receptors in rats, can be expanded and extended to enable its use: (i) with endocytotic receptors other than the p75 receptor; (ii) with endocytotic surface molecules other than receptors; (iii) with endocytotic receptors that are present in species other than just rats, including human receptors; and, (iv) with endocytotic receptors that are present on specific types of cells and tissues other than neurons (such as, for example, receptors that normally are found in significant numbers only on the surfaces of cells in kidneys, livers, lungs, hearts, etc.).

This type of work has been done before in a number of cases, because once the DNA sequence that encodes a particular type of human receptor protein is known, that human gene sequence (or any portion thereof) can be used to transform animals of a different species, such as mice or rats. The genetically transformed animals will then express the human receptor protein, having the exact same human amino acid sequence. As just one example, the human receptor protein that enables polio viruses to infect certain types of motor neurons, in humans and certain other primates, was used to transform mice. This allowed the transformed mice to be used as inexpensive animal models, for studying polio and polioviruses.

In a similar manner, as an example of how that type of genetic engineering can be adapted to enable in vivo screening as disclosed herein, the following series of steps can be carried out, by skilled artisans, using DNA sequences and other reagents and methods that are already known and available in the art:

1. The human homologue of the p75 gene, which has been fully sequenced (Johnson et al 1986) can be placed in a chimeric gene, under the control of the p75 gene promoter normally found in mice or rats;

2. This chimeric (mice or rat promoter/human coding) version of the p75 gene can

then be used to genetically transform selected types of mice or rats, such as strains of mice that have a "knockout" mutation which prevents them from properly expressing the p75 receptor; such strains are available from Jackson Laboratories (www.jax.org, or www.jaxmice.jax.org), as stock number 002213 (strain name B6.12954-Ngfr^{tm1Jae}).

3. The transformed mice or rats will express the human version of the p75 receptor protein, and human p75 receptors will appear in the same locations where the rat p75 receptor protein normally exists, including on the surfaces of sciatic nerve fibers that extend outside the blood-brain barrier;

4. A ligand library (such as the scFv phage display library, or the PhD-C7C phage display library) is then screened, using the same procedures disclosed herein, to identify candidate phages that will undergo endocytotic uptake and retrograde transport, through a process that is mediated by binding of the ligand domain of a phage particle to the human version of the p75 receptor.

5. Alternately or additionally, ligands that have been discovered and identified to enter cells through the rat p75 receptor protein can be tested, *in vitro*, to determine whether they will also enter cells that have human p75 cell receptors (such as on a human neuroblastoma cell line that grows readily in suspension culture and that expresses the natural version of human p75;

6. Alternately or additionally, if the endocytotic efficiency of a ligand that readily enters rat neuronal fibers through rat p75 receptors is tolerable but not very high, when it interacts with human p75 receptors, then that particular ligand can become the starting compound in a process that will (i) use site-directed or random mutagenesis to create numerous analogues of the rat-p75-binding ligand, and (ii) use *in vitro* screening to identify and evaluate promising analogues that can readily enter cells through human p75 receptors.

These are just a few examples of how the *in vivo* screening methods disclosed herein can be adapted for use in discovering, isolating, and analyzing ligands that will enable efficient transport of passenger or payload molecules into human cells, for use in human medicine, diagnostics, analysis, and research.

Molecular Complexes and Methods Enabled by This Invention

The true value of the screening methods disclosed herein comes not from the act of identifying particular ligands that can activate and drive endocytotic internalisation, but from the subsequent ability to incorporate and use those selected ligands, in "molecular

complexes" that can be used for medical, diagnostic, and similar purposes.

As used herein, the term "molecular complex" refers to a molecular assemblage that includes at least two distinct components: at least one ligand component, and at least one passenger or payload component.

In order to fall with the claims that refer to such ligands or to molecular complexes which include such ligands, a ligand component must meet two criteria, as follows.

First, the ligand component must have been identified by an *in vivo* selection process as disclosed herein (i.e., to be covered by a claim such as claim 1, the ligand component must have been identified by a process of *in vivo* selection that required, at a minimum, endocytotic uptake into neuronal fibers, for such selection to occur). This type of identification is an essential step, in the screening methods of invention, and in the molecular complexes that can be formed using ligands that were in fact identified by this method. To illustrate this fact, it can be presumed that a phage library containing billions of candidate ligand polypeptides (such as the scFv library, which was used and screened as described in Examples 28 and 29) does indeed contain hundreds, thousands, or possibly even millions of phage particles that are indeed carrying candidate ligand sequences that are quite capable of serving as potent, specific, effective endocytotic ligands, which could be used to carry passenger molecules into cells having p75 receptors on their surfaces. However, those hundreds, thousands, or even millions of phage particles which have that theoretical potential, in that huge library, are surrounded by billions of other phages that would be totally useless for that purpose, and that would provoke all kinds of unwanted responses if coupled to passenger molecules and injected into an animal or human that needs medical treatment.

Obviously, the screening and processing steps that are required to identify and isolate those phages which carry ligand sequences having a known and useful endocytotic activity is an absolutely critical step, in creating molecular complexes which can actually accomplish desirable and useful medical, analytical, or similar results.

The second requirement that applies to the ligand components that are used to transport passenger or payload components in molecular complexes, as described and claimed herein, is this: the molecular complex, which includes a ligand component that was initially identified by the *in vivo* screening process disclosed herein, must be able to actually enter targeted types and classes of cells which have endocytotic surface molecules to which the ligand component will bind. If such a molecular complex cannot enter at least one class

of such cells, then that molecular complex is not covered, and is not intended to be covered, by the claims herein.

It should be clear, however, that once a particular ligand has been identified which can enter cells through a particular and targetable class of endocytotic surface molecules (and once the amino acid sequence of that particular ligand is known, if that ligand is a polypeptide), then that particular ligand can be synthesized in any desired quantity, and it can be used as an endocytotic transport system to carry a wide range of useful "passenger" or "payload" molecules into targeted cells. Accordingly, after such a ligand component has been identified by means of the new and powerful *in vivo* screening methods disclosed herein, then the use of that ligand, as an endocytotic transport component, in molecular complexes that also contain "passenger" or "payload" molecules, is not limited to any one specific type or class of passenger or payload molecule.

The terms "passenger molecule" and "payload molecule" are used interchangeably herein, to refer to the portion of a molecular complex (i.e., containing a ligand as set forth above) that will perform one or more useful functions, or exert one or more useful effects, after the passenger molecule has entered a targeted cell containing an endocytotic surface molecule to which the ligand component will bind. These terms are intended to be construed broadly, and in general, a passenger or payload molecule must be interpreted by recognizing how these same terms are used in other modes of transportation. Cars, buses, trains, airplanes, and bicycles are all useful, because they can carry passengers, at speeds and over distances which simply cannot be achieved, on a practical level, by other means of transportation. Similarly, freight trains, 18-wheelers, and tanker trucks and boats are useful because they can carry freight, which can be regarded as the payload whenever a trip is being made to an intended destination. Cars, buses, trains, airplanes, bicycles, and boats are highly useful and valuable modes of transport, not just because they can carry one particular person to one particular place, but because they can be adapted and used to carry numerous types of passengers or freight to numerous selected and targeted destinations.

Accordingly, passenger or payload molecules, as disclosed and contemplated herein, should be interpreted broadly, and include but are not limited to each of the following major classes:

(1) DNA segments that are part of genetic vectors that are intended to genetically transform animals, or to medically treat humans in need of genetic therapy. Indeed, one of the first and foremost goals of this entire line of research was to identify and create cell-

targeting components that could be used to create new classes of genetic vectors that can be used to specifically target and transform only certain particular types of cells, without disrupting the status or activities of other cell types in ways that would greatly increase the risk and severity of unwanted side effects.

(2) therapeutic and/or diagnostic compounds (including pharmaceuticals, imaging compounds, etc.), for use in human or veterinary medicine.

(3) analytical compounds, reagents, and other substances that would be more useful, in industrial research and similar endeavors, if they could be transported efficiently into targeted classes of cells.

Finally, it should also be noted that a molecular complex which contains both a ligand component, and a passenger or payload component, must also have some effective means for coupling and holding those two components together, to form a complex that will hold together at least until the passenger or payload component has been successfully pulled inside a targeted cell. In some cases, depending on the passenger or payload component, it may be possibly to couple the passenger or payload component directly to the ligand component, by means of a direct covalent bond, or by means of a "coordinate" bond (this term refers to a class of molecular bonds having levels of strength and/or stability that fall somewhere between covalent bonds, and ionic attractions). However, if a passenger component is bonded directly to a ligand component, it is likely that this type of molecular complex may not be optimal, for at least some types of intended uses, because it often will be necessary to release the passenger component from the ligand component, after the molecular complex has entered a targeted cell, so that the passenger component can then carry out its intended function without having the ligand component still attached to it. By way of analogy, this is comparable to saying that a car, truck, or bus will be substantially more useful, if is it provided with doors that will allow passengers to leave the vehicle, once the vehicle arrives at an intended destination.

Accordingly, most types of molecular complexes that contain ligand and passenger components as disclosed herein preferably should also contain a suitable "coupling component", which will attach the ligand and passenger components to each other by a suitable means that will balance two different needs: (i) it must be sufficiently strong and stable to enable the molecular complex to remain intact, while the ligand component is performing its role and helping pull the passenger component into a cell interior; and, (ii) in many cases, unless the passenger component can exert its desired effects while still

coupled to the ligand component, the coupling means should provide some type of structure or mechanism that can allow the passenger component to eventually be released or detached from the molecular complex, after the molecular complex has successfully entered a cell.

This patent application is not an appropriate forum for an exhaustive review of candidate coupling components that can achieve and/or balance those two competing goals. A variety of such candidate coupling components are known to those skilled in the art of drug delivery, and any such candidate coupling component can be evaluated for use in a particular molecular complex as disclosed herein, after the complete details of the ligand component, the passenger component, and the targeted cell type are all known.

Some of the broad classes of coupling compounds should be briefly mentioned, to provide an overview and working introduction to the range of options that will be available when a particular type of molecular complex is being designed to optimize a combination of a known ligand, a known passenger molecule, and a known targeted cell type:

1. crosslinking agents that form covalent bonds by using relatively non-specific reactive groups, such as glutaraldehyde and other compounds that contain two aldehyde or other non-specific reactive groups at opposite ends of a spacer chain having a controlled length.

2. crosslinking agents that form covalent bonds, but only with specific molecular groups. These include "sulfo-SMCC", described in Example 24, which is used to crosslink an end of a DNA strand to a lysine residue in a polypeptide, for purposes such as genetic vectors and affinity purification.

3. affinity binding agents, which can have very high levels of tightness and avidity (as occur between two polypeptides called biotin and avidin, mentioned in Example 27), and which can have virtually any desired lower but still substantial level of tightness and avidity (which can be controlled by various means, such as by controlling the elution conditions during an affinity purification procedure).

4. coupling agents that use ionic attraction and/or hydrogen bonding to hold two components together. Since ionic attraction and hydrogen bonding are not especially strong, these types of agents typically involve compounds that contain multiple ionic charges, all of the same polarity, packed together in a fairly close arrangement. Examples include polylysine, polyethylenimine, and other positively-charged compounds that will attract and associate with negatively-charged phosphate groups in the backbones of strands of DNA and RNA.

5. special types of connector molecules that are designed to weaken and break, when a molecular complex is subjected to acidity (such as occurs in lysosomes, which are acidic digestive organelles inside cells). These types of connector molecules are described in US patents 4,631,190 (Shen et al 1986) and 5,144,011 (Shen et al 1992).

This is just a brief overview, and other types of connector molecules are also known to those skilled in the art. Nevertheless, it should be adequately clear that various known options with a wide range of strength, stability, and other traits are available, for coupling passenger components to ligand components.

Overview of Examples 22 - 32 (In Vivo Screening Methods)

Because of the complexity of the methods that are described in Examples 22-31, and of the specific types of phages and cells that were used as reagents in those tests, and because some of the test procedures that were eventually settled upon were chosen after the Applicants analyzed prior efforts that did not succeed, this section is intended to offer an overview and a narrative summary of the examples, and of how their information is organized.

Example 22 describes three types of bacteriophages that were used. These included: (1) M13KO7 helper phages, which carry no endocytotic ligands; (2) a phage display library known as the scFv library, which contains roughly 13 billion different phagemids, each of which carries a candidate ligand that normally appears in human antibodies; and (3) a phage display library known as the PhD-C7C library, which carries small foreign polypeptide sequences that contain 7 amino acid residues, which were sequenced together randomly, using "combinatorial chemistry".

Example 23 describes the host cells (mainly the TG1 strain of *E. coli* cells) that were used, and it describes several techniques that were used with numerous phage populations, to amplify and titer those particular phage populations.

M13KO7 helper phages are described first, in Example 22, even though they do not carry ligand polypeptides, because they were used to create antibody-phage conjugates. These conjugates were prepared to display copies of the MC192 monoclonal antibody, which is known to be internalised by rat p75 receptors, crosslinked to the surfaces of the helper phages. These antibody-phage conjugates were tested first, and shown to be internalised by sciatic nerve fibers. Accordingly, these established a set of tools (comparable to probe drugs) that enabled the Applicants to work out the concepts and

details of an effective approach that allows *in vivo* screening for endocytosis, in rats by manipulating sciatic nerve fibers. The methods that were used to crosslink the antibodies to the helper phages are described in Example 24, and the methods that were eventually developed to achieve endocytosis and retrograde transport of the antibody-phage conjugates, in sciatic nerves, are described in Examples 25 and 26. The methods and reagents that were used to create photographic proof of endocytosis and retrograde transport of those antibody-phage conjugates, as shown in FIG. 8 of this application, are described in Example 27.

After the antibody-phage conjugates were used to develop a set of consistent procedures for achieving reliable uptake of phage particles via p75 receptors, the Applicants began testing a phage display library known as the scFv library. The major traits of that library are described in Example 22. Very briefly, it contains a huge number (roughly 13 billion) of foreign gene inserts that were initially obtained from human B-cells. These gene inserts encode the "variable fragments" of a wide range of human antibodies, from people of different ancestries. The initial *in vivo* screening tests that were done with this library did not provide consistent results. Therefore, the Applicants wrestled with those problems, and eventually settled on a process of pre-screening the scFv library, *in vitro*, using a technique called "biopanning", described in Example 28. Very briefly, this pre-screening involves p75 polypeptides that have been immobilized on a hard plastic surface. Phage particles that bind to these immobilized p75 polypeptides were selected by this step, and used for subsequent *in vivo* screening, which provided much more consistent results that could be understood and interpreted. These procedures, and the results that were obtained, are described in Example 29.

Subsequently, the Applicants also tested their *in vivo* screening method on a second phage display library, called the PhD-C7C library. As summarized in Example 22, this library was created by combinatorial chemistry, and contains short polypeptide segments (with 7 amino acid residues) that were randomly generated, and inserted into the pIII coat proteins of the phages. These tests, and their results, are described in Example 30.

The results of all of these screening tests confirm that *in vivo* screening of phage libraries, to select particular phages that are internalised and transported by neuronal fibers, is indeed a practical and effective way of identifying and isolating, from a large display library, particular phages that happen to carry ligand components that can activate and drive the process of endocytosis into nerve fibers.

These results, taken together, also confirm that stochastic processes are involved,

which rely on probability, and on the sizes of the populations that are being challenged and tested in a highly specialized set of tests. The assertion and claim herein is not that this type of selection process will succeed, in each and every screening attempt or round. Instead, the assertions and claims made herein center on the fact that this selection process, which uses *in vivo* tests on living animals in ways that were not previously known or possible, can be used (in repeating cycles, if desired) to identify, select, and isolate a small number of clonal display phages that will successfully enter into and be retrogradely transported by neuronal fibers, from among a potentially huge and/or random starting library or repertoire.

These titering and photographic data also clearly demonstrate that this approach enables an *in vivo* screening method that can effectively identify ligand molecules and ligand fragments that can activate and drive the process of endocytosis, even when coupled to large molecular complexes. Based on those results, this type of *in vivo* screening method can enable researchers to identify such molecules (referred to herein as "endocytotic ligands"), and use them as part of a molecular transport system (which can also be called a carrier, vehicle, etc.) that can be used to transport "passenger" or "payload" components into cells. Such passenger components can include, for example, DNA segments that are part of genetic vectors, drug or diagnostic molecules that can provide therapeutic, diagnostic, or other medical benefits, and analytical compounds that would be more useful, in industrial research and similar endeavors, if they could be transported efficiently into cells.

It should also be disclosed that, as this patent application is being written and filed, none of the nucleotide gene sequences that encoded the polypeptide ligands that performed well in the *in vivo* screening tests are yet known, and none of the amino acid sequences of those polypeptide ligands are known. The laboratories of the Applicants herein (which are located in Australia) do not have the types of machines that are used to determine nucleotide or amino acid sequence information. Accordingly, the Applicants shipped copies of a number of selected phages that performed well in their *in vivo* screening tests, to an outside contract laboratory which is equipped to determine those sequence data. However, the resulting data have not yet been received, and those sequences are not yet known, as of the day this patent application is being filed.

USING GENETIC VECTORS FOR DRUG ADMINISTRATION/DELIVERY INSIDE BBB

The invention may be used by administering, into or onto tissue which is not protected by the BBB, one or more copies of a gene vector that can transfect one or more types of BBB-straddling neurons. Clearly, the range of preferred and potential modes of administration will depend on the type of BBB-straddling neuron that is to be transfected by the vector, and the type and location of the peripheral projection that is to be contacted by the vector. As described above and in the examples, administration to olfactory receptor sensory neurons can be via nasal instillation; administration to nocioceptive neurons can be via cutaneous, subcutaneous, or possibly intramuscular injection, and possibly by topical administration (which can be accompanied by one or more agents or techniques that will increase epidermal penetration and tissue permeation, as described above); administration to lower motor neurons innervating the skeletal musculature can be via intramuscular injection; and, administration to the lower motor neurons of hypoglossal nucleus can be by injection into the muscles of the tongue.

It will be apparent to those skilled in the arts of medicine or neuroscience that there exist other ways to administer gene vectors to peripherally-projecting neurons. It also will be apparent to such persons that: (i) other types of neurons that straddle the BBB (including various types of sensory and motor neurons, as well as pre-ganglionic neurons of the sympathetic and parasympathetic systems) offer potentially useful targets for transfection; and, (ii) the known features and traits of the anatomy and structure of any such class of neuron will allow skilled neurologists and researchers to develop various methods for administering gene vectors to such neurons.

MODULATION OF ENDOCRINE AND PARACRINE HORMONAL SYSTEMS

It should also be recognized that, by enabling non-invasive delivery of specific gene-encoded polypeptides to cells, systems, and regions within the brain, this invention may also be able to provide new and previously unavailable methods and approaches to controlling or modulating various types of "downstream" effects or activities, such as by increasing or suppressing the release of various types of endocrine and/or paracrine hormones by various glands or organs, either in the CNS-protected brain tissue (such as the pituitary and pineal glands), or in other parts of the body (such as the thyroid, thymus, adrenal, or other glands, or in the pancreas, reproductive organs, etc.).

This patent application is not the appropriate location for a detailed analysis of the endocrine or paracrine systems, and the comments below are intended solely as a very brief introduction and overview. For more information on the endocrine and paracrine systems, good overviews are provided in nearly any good textbook on physiology, and more information is contained in numerous full-length textbooks, such as Wilson & Foster 1992, Barrow & Sleman 1992, Brown 1993, DeGroot 1994, etc. Recent review articles are not especially helpful in establishing a working knowledge of the endocrine or paracrine systems, since they focus mainly on problems (such as glandular tumors, hormone disruptors such as pesticides, etc.), interventions (surgical or drug), or interactions between hormone systems and other systems such as immune responses; however, review articles offer a good base of information when the goal is to move beyond a working knowledge of the endocrine or paracrine systems, and into the realm of potential interventions for purposes such as therapy of human disorders or malformations, or livestock breeding.

One of the crucial components of the endocrine system is the pituitary gland, which sits at the base of the brain, suspended from a region of brain tissue called the hypothalamus. The anterior lobe or gland of the pituitary is known to release at least six different hormones, and the release of each of these hormones is either triggered or suppressed by an "upstream" hormone, called a hypothalamic hormone. These hormonal systems (or pairings, relationships, etc.) include the following:

1. a hypothalamic hormone called thyrotropin-releasing hormone (abbreviated as TRH; formerly called thyroid-stimulating hormone releasing hormone) causes the pituitary to release a hormone called thyrotropin (formerly called thyroid-stimulating hormone, or TSH);

2. a hypothalamic hormone called corticotropin-releasing hormone (CRH) causes the pituitary to release adrenocorticotropin;

3. a hypothalamic hormone called growth hormone releasing hormone (GHRH) causes the pituitary to release growth hormone (also called somatotropin, and often referred to as hGH or HGH in the case of human growth hormone);

4. a hypothalamic hormone called growth hormone inhibitory hormone (GHIH, also called somatostatin) inhibits the release, by the pituitary, of growth hormone (somatotropin);

5. a hypothalamic hormone called gonadotropin releasing hormone (GRH or GnRH) causes the pituitary to release two types of "gonadotropic" hormones, called luteinizing

hormone, and follicle-stimulating hormone; and,

6. a hypothalamic hormone called prolactin inhibitory hormone (PIH) inhibits the release, by the pituitary, of a hormone called prolactin.

By administering a genetic vector that will cause an increase in the concentration of one of the above-listed hypothalamic hormones inside BBB-protected brain tissue (it should be noted that a transient rather than permanent increase can be achieved by the methods disclosed herein, and transient increases are generally presumed to be preferable in therapeutic treatments of human medical or developmental disorders), it is likely to be possible to cause, in a controllable manner, either stimulation or inhibition of the release of a "downstream" or "dependent" pituitary hormone. Accordingly, as a result of that type of triggered and/or targeted pituitary stimulation or inhibition via a genetic vector, it is possible to stimulate, inhibit, or otherwise modulate the same types of physiological effects that are caused by the release, or inhibition, of the pituitary hormones.

As an alternate approach, it may be possible in at least some cases to stimulate or inhibit a targeted endocrine or paracrine system by administering a genetic vector that directly encodes a pituitary hormone, rather than its "upstream" hypothalamic hormone. Along these lines, radiolabelled tracer studies have shown that at least some types of proteins which have been delivered into the CNS by direction injection into a brain ventricle are cleared fairly rapidly from the CNS into the blood circulation (e.g., Ferguson 1991). Therefore, if the rate of delivery of a hormone-type polypeptide into BBB-protected CNS tissue is sufficiently high, some of those hormone polypeptide molecules will diffuse into circulating blood, and will be distributed systemically.

With regard to delivering hormonal or other polypeptides into the brain with the intent of causing the polypeptides to contact specific regions, cells, or structures within the brain, it should be borne in mind that this invention may be able to offer, in at least some cases, an approach which will allow targeted delivery in ways that have not previously been available. Most prior art methods of delivering neuroactive molecules (such as neurotrophic factors) into the CNS appear to assume that the endocrine model of drug delivery is the appropriate method for delivering such molecules. Evidence that this assumption is prevalent is seen in numerous animal studies, as well as limited human clinical studies in which recombinant neurotrophins are injected or infused into cerebrospinal fluid (typically into the lateral ventricles). These drug administration approaches are based on the assumption that the flow of cerebrospinal fluid within the brain and spinal cord represents

an internal CNS circulation, analogous to the circulation of blood within the periphery.

However, that assumption is unwarranted, because the flow of CSF in the CNS is more analogous to the lymphatic system, in the periphery, than to blood circulation. Like the flow of lymph fluid, the flow of CSF is more of a uni-directional drainage, rather than a re-circulation of fluid.

Accordingly, when neuroactive molecules (such as neurotrophic factors) exert their physiological effect, not in an endocrine (system-wide) manner but rather in a paracrine (localized) manner, an appropriate drug delivery method preferably should not involve systemic administration, such as by intravenous infusion or injection, both because of (i) high levels of wastage of highly expensive drug compounds, and (ii) the potential for unwanted adverse effects, when systemically-injected molecules react with various cells or organs other than the desired targets. Instead, a more narrowly focused and targeted system should be used, if available.

This invention appears to offer a substantially improved method of drug delivery, which in some respects emulates paracrine delivery. This method can achieve or at least promote localized and focused delivery of drug to a target cell or region (especially when compared to other methods, such as intravenous injection) by transfecting only certain selected populations of BBB-straddling neuron(s), which will subsequently release polypeptides in a limited and desired "secretion zone".

The ability to use gene therapy to achieve sustained drug delivery has been recognized in the art, as evidenced by numerous studies involving transplant into the CNS of cells that have been genetically engineered to secrete particular recombinant molecules, such as neurotrophins. However, nearly all such studies illustrate or imply that the desired intention is to achieve an endocrine-like form of drug delivery, with the transplanted cells secreting drugs in a manner that causes or allows systemic distribution. By contrast, this invention discloses an entirely different form of genetic therapy, which can achieve a paracrine-like, localized delivery of therapeutic polypeptides to a specific cluster and/or type of neuron within the BBB, by transfecting a limited number of neighboring cells that straddle the BBB.

SELECTIVE MODULATION OF NEURONAL SYSTEMS

The invention enables development of completely new approaches to treating disorders of the nervous system, and the disclosed paracrine-like method of drug delivery

enables development of new processes or methods for selectively modulating the function of particular systems of neurons within the CNS.

Physiologically, the function of systems of neurons within the central nervous system can change, in response to functional changes in the neurons that penetrate through the BBB. For example, changes in the electrical activity of nocioceptive neurons that penetrate through the BBB cause changes in the electrical activity of second order sensory neurons and the associated system of CNS neurons involved in sensation of pain and response to that pain. The anatomical connections between the neurons that project through the BBB, and neurons that reside wholly within the BBB, are not fixed and static; instead, the nature, number, and distribution of these connections can change, both over time and in response to various types of events (and often resulting in downstream changes in still other systems of neurons within the CNS). In other words, the systems of neurons within the CNS are plastic, changeable, and responsive to functional changes in the neurons that penetrate through the blood brain barrier.

Based upon that physiological fact, it is believed that in at least some cases, this invention may render possible to selectively modulate and alter the structure and functioning of at least some types of neuronal systems within the CNS, by altering the patterns of innervation (including strength, number, and distribution) of synaptic connections between transfectable peripherally-projecting systems, and targeted neuronal systems within the CNS.

As an example, it is well known in the field that neurotrophic factors are intimately involved in the plastic changes in synaptic density and innervation pattern, in CNS systems, in response to changes in electrical activity, such as the number and frequency of nerve impulses that arrive from other neurons located closer to the periphery. Physiologically, these neurotrophic factors act in what has been described above as a paracrine-like manner. It follows that the paracrine drug delivery approach disclosed herein can be used to selectively modulate the nature and extent of connections between one or more peripherally projecting neurons, and the CNS neurons or neuron systems with which they interact. That is, by using gene vectors to transfect BBB-straddling neurons, it will be possible (in at least some cases) to alter the nature and extent of the connections between those BBB-straddling neurons, and CNS neurons lying wholly within the BBB, and thereby selectively modulating one or more densities, functions, or other traits of the interacting CNS neuronal systems.

As examples, this principle can be illustrated by two preferred embodiments: (i)

administration of recombinant anti-NGF, via transfection of nociceptive neurons that straddle the BBB, can modulate the BBB-protected neuronal systems that are involved in the perception of pain; and, (ii) administration of recombinant NT-3 or GDNF, via transfected BBB-straddling spinal motor neurons, can modulate the CNS neuronal systems involved in voluntary control over motor function.

TREATMENTS FOR NEUROLOGICAL DISORDERS

This invention is likely to become useful for treating neurodegenerative disorders of the CNS (such as Alzheimer's disease), by methods which include delivering neurotrophic or neuroprotective factors to the neurons at risk of degenerating. It is believed, for example, that Alzheimer's disease probably can be treated in a useful manner by administering neurotrophic factors (such as nerve growth factor) into the CNS. In accord with that goal, this invention allows neurotrophic factors to be delivered, in a relatively focused and targeted manner, to basal forebrain cholinergic neurons that are at risk of degenerating in patients with Alzheimer's disease. In at least some patients, this type of treatment may be able to help slow, and potentially even halt, the neurodegenerative process.

This invention is also likely to become useful for treating trauma or injury to the CNS (such as that which occurs in head injury), by delivering neuronal growth factors (such as neurotrophic factors) to injured and surviving neurons. As just one example, GDNF, which acts on cortical motor neurons (which are frequently damaged in stroke or sometimes head trauma), can be delivered to these neurons by methods disclosed herein. Its potential benefits are described in various articles such as Schacht et al 1996.

This invention may also become useful for treating some cases of learning or memory dysfunction, such as occur in aging, dementia, after brain trauma or injury, and after various types of major surgery, especially surgery involving a cardiopulmonary bypass machine. Such trauma and insults often lead to loss of function within one or more regions of the CNS. Restoration of function, if it can be achieved, apparently requires and involves compensatory changes in the organization of the CNS, typically including the sprouting and outgrowth of various affected neurons, and the establishment of functional synapses on other neurons. These types of "neuroplastic" processes have been demonstrated both in animal studies, where a digit or limb was severed, and in human cases, where a stroke victim regained substantial use of a paralyzed limb by undergoing therapy in which one or

two healthy limbs were strapped to the body and immobilized, while the patient did repetitive exercises which actively and aggressively challenged the patient to begin moving and using the impaired limb once again, building upon and expanding the range of motion and control that remained in the limb after the stroke.

Neurotrophic factors can play important roles in such processes (e.g., Lo 1995), and administration of NGF into the brain has been shown to enhance various CNS activities and functions, such as memory and learning (e.g., Fischer et al 1987 and 1991).

Accordingly, this invention provides a major avenue for expanding and enlarging upon that type of highly useful therapy, by allowing NGF and other neurotrophic and neurostimulatory factors to be delivered through the CNS and into the brain, using improved delivery systems.

This invention is also likely to become useful for treating disorders due to excitotoxic damage of neurons, or resulting from diseases or injuries that involve ischemia (inadequate blood flow, as occurs during a stroke or cardiac arrest) or hypoxia (inadequate oxygen supply, as occurs during drowning, carbon monoxide poisoning, etc.) or traumatic head injury. In animal studies, NGF infusion can slow or reverse the retrograde atrophy of cholinergic cell bodies and fiber networks and other changes in the cholinergic system that are caused by infarction or measured by infarct volumes or severity (e.g., Cuello et al 1992). Administration of NGF (or induction of NGF synthesis *in vivo* by clenbuterol) has been shown to reduce infarct volume in rat models of permanent middle cerebral artery occlusion (Semkova et al 1999). Other animal data suggests that NGF is able to act after a brain insult to block progression of neuronal damage (e.g., Guegan et al 1998). Since this invention is likely to prove useful for administering NGF after a stroke or other brain injury or insult, it likely will be able to reduce the extent and severity of subsequent neuronal loss.

The present invention further relates to methods for treating disorders of sensory function by modulating the function of the sensory neuron and/or the nerve cells which make synaptic contact with it in the CNS. For example, severe and persistent pain involves both nocioceptive neurons and CNS (spinal or brain) changes, and it has been reported that intrathecal administration of anti-NGF can reverse these types of changes, and alleviate the pain (e.g., Christensen et al 1996 and 1997). This invention allows the function of the sensory system to be modulated by delivering polypeptides (such as antibodies which will bind to and inactivate NGF, or which will occupy and block NGF receptors) which act on

one or more components of the sensory system, to alter its physiological function.

This invention can also be used for modulating neuronal physiology by delivery and expression of neuropeptide genes, such as genes that express polypeptides that can block and suppress pain (such as so-called "endorphins"); genes that express growth factors; genes that express polypeptides to promote regeneration or prolong the life-spans of cells; and genes that express toxic polypeptides, such as to kill tumor cells.

Beyond that, this invention provides an approach that can be adapted to treatment of various types of CNS-related neurological disorders or deficiencies which are correlated with either too little or too much of some particular polypeptide. This can be accomplished by using this method to deliver, into BBB-protected CNS tissue, either: (i) a polypeptide which provides an additional quantity of a polypeptide, to reduce or eliminate a deficiency; or, (ii) a polypeptide which blocks, antagonizes, or otherwise suppresses a certain molecule, receptor, or reaction, thereby helping to controlling a CNS disorder that is caused or characterized by too much of a particular molecule.

EXAMPLES

The Examples below are organized as follows:

Examples 1-8 relate to delivering neuron-stimulating polypeptides to neurons which lie wholly within the BBB, by transfecting olfactory receptor neurons, using vectors that carry genes which encode such polypeptides. For purposes of illustration, human NGF is used as the prototypic polypeptide; as will be recognized by those skilled in the art, genes which encode other forms of NGF (such as mouse, other rodent, or simian NGF, or any mutated, epitope-tagged, fragmented, or other form of NGF which may be of interest in medicine or research) may alternately be used.

Examples 1-7 describe how NGF (or other neurotrophic or similar polypeptides) can be delivered to cholinergic neurons in the basal forebrain of a laboratory animal, such as a rat. Examples 1-4 contain a complete embodiment, divided into various sequential steps. Example 1 describes the assembly of the vectors; Example 2 describes administration of those vectors to the nasal sinuses; Example 3 describes methods of monitoring delivery of the polypeptide through the blood brain barrier; and Example 4 describes methods of measuring the physiological and behavioral effects of such treatments on lab animals.

Following that "start to finish" description, Examples 5-8 describe delivery of an

NGF-encoding or similar genes into olfactory receptor neurons, using different types of vectors. Example 5 describes vectors derived from herpes viruses; Example 6 describes liposome vectors; Example 7 describes vectors with ligands that bind to endocytotic receptors on neuron surfaces; and Example 8 describes vectors that use "transneuronal" polypeptides that can promote transport of the entire vector from one neuron to another.

Examples 9-13 involve a different approach, using gene vectors that encode nerve suppressing (rather than stimulating) factors; a polypeptide called "anti-NGF", which binds to and inactivates NGF, is used as illustration. These vectors are injected into skin or muscle regions, in order to transfect nocioceptive (pain-signalling) neurons, in areas that suffer from unwanted and excessive pain signals (often called neuropathic pain, or allodynia). By suppressing over-active pain signalling circuits, this approach can help reduce and control neuropathic pain.

Examples 14-20 describe a third major line of approach, in which genetic vectors carrying genes that encode nerve-stimulating factors are injected into muscle tissue that is impaired due to a stroke, spinal injury, etc. These types of impaired muscles often suffer from a lack of (or impairments in) voluntary control, caused or aggravated by a loss of properly functioning connections between upper motor neurons (which lie wholly within the BBB) and lower motor neurons (which straddle the BBB). Transfection of lower motor neurons in such impaired muscles, using genes that encode nerve-stimulating factors, can help expand, repair, and reconnect the damaged motor control networks, by means such as establishing new and additional connections between the lower and upper motor neurons, and/or by increasing synaptic activity levels between those classes of neurons, thereby reestablishing proper innervation and CNS control over such muscle systems.

Example 21 describes transfection of certain types of motor neurons located inside the tongue. This route of administration deserves special attention, since it offers a route for delivering polypeptides into certain portions of the brainstem.

EXAMPLE 1: CONSTRUCTION OF ADENOVIRAL VECTOR FOR TRANSFECTING OLFACTORY RECEPTORS WITH NGF GENE, TO DELIVER NGF TO CHOLINERGIC NEURONS IN THE BASAL FOREBRAIN

Methods for preparing non-pathogenic vectors derived from adenoviruses that cannot replicate, except in genetically engineered host cells that exist only in laboratories, have been published in articles such as Graham and Prevec 1995. Methods for creating gene

constructs which are small enough to be carried and delivered by adenoviral vectors, and which will express NGF (or some other CNS-active polypeptide, such as GDNF, NT-3, CNTF, or BDNF, or any of numerous other polypeptides such as listed in Table 1) at significant levels, inside transfected human neurons, are described in articles such as Romero et al 2000, Baumgartner and Shine 1998), Dijkhuizen et al 1997, and Gravel et al 1997. Methods for propagating, purifying, concentrating, and titering adenoviral vectors carrying such gene constructs can be found in publications such as the chapter by Engelhardt (pp. 169-184) in *Methods in Molecular Medicine: Gene Therapy Protocols* (P. Robbins, ed., 1997).

The gene construct that will drive expression of the NGF polypeptide (or other selected CNS-active polypeptide) will require proper selection of all relevant portions of the gene. As used herein, terms such as "gene" or "gene construct" normally refer to a complete and functional transcription unit, which can be manipulated under laboratory conditions using known procedures, and which, if transfected into a BBB-straddling neuron, is capable of: (i) causing the normal transcription and translation mechanisms inside the neuron cell body to synthesize the polypeptide encoded by the gene, and (ii) instructing the cell to appropriately process and secrete the mature polypeptide from dendrites, processes, synapses, or other terminals located inside the BBB.

A number of variants and enhancements of the simple "plain vanilla" type of gene constructs are known to those skilled in the art, including a number of variants and enhancements described in the Detailed Description section. Any such variants or enhancements are included within the terms "gene" or "gene construct" as used herein.

The complete amino acid encoding sequences (without introns) of human NGF, mouse NGF, and certain other forms of NGF, have been published and are known and available, in easily manipulated plasmid form, from various researchers; alternately, they can be created using well-known techniques and published information.

Also, if a polypeptide having an unusual "epitopic tag" sequence (such as c-myc) is desired, then the coding portion of the gene construct must include the DNA sequence that will encode the "epitopic tag" sequence. The inclusion of an epitopic tag may assist in demonstrating, refining, and otherwise enhancing the invention, in situations where epitopic tag sequences can assist researchers to distinguish between exogenous polypeptide molecules that are encoded by a genetic vector, and endogenous (native) polypeptides that are present naturally within the BBB of test animals or patients.

The other crucially important part of a gene construct is the gene promoter, and numerous known gene promoters are known and available which can drive gene expression in olfactory receptor neurons. Examples of "selective" gene promoters that are known to drive gene expression specifically in olfactory receptor neurons include promoters identified in the olfactory marker protein gene (Servenius et al 1994) and the M4 olfactory receptor gene (Qasba and Reed 1998). Use of such selective promoters can help minimize the chance that undesired or uncontrolled expression of the foreign gene will occur in surrounding cells or tissue.

Alternately, various types of viral and other promoters which are known to be unusually strong promoters in mammalian cells can be used if desired, for purposes such as inducing the highest practical levels of expression of NGF (or other CNS-active) polypeptide in transfected cells. Examples of such strong promoters include the early gene promoter from cytomegalovirus, and the late gene promoter from simian virus-40. Inducible gene promoters can also be used if desired, so long as the inducing factor which activates the selected promoter can be administered in a way which ensures that it will be transported into transfected neurons in adequate quantities.

As noted above, for simplicity of discussion herein, the term "gene promoter" as used herein includes the so-called "TATA box", and the sequence of about 25 bases between the TATA box and the actual start of transcription.

Once the promoter and coding sequence of the gene construct have been chosen, various other portions of the gene construct can be chosen to enhance expression, packaging, transport, secretion, and/or performance of the vector-encoded polypeptide. These gene portions can include, for example: (i) a DNA sequence which will encode a "leader" or "signal" peptide sequence, which will instruct the transfected neuron to appropriately process and secrete the vector-encoded polypeptide (or a "mature" form from which the leader or signal sequence may be removed); (ii) a DNA sequence that will be transcribed into an mRNA sequence that will serve as a non-translated "tail" region, which will follow the "stop" codon in the mRNA, to facilitate translation and release by ribosomes; and, (iii) a transcription terminator sequence, which will direct RNA polymerase to truncate the mRNA strand when the DNA sequence is being transcribed into mRNA. These types of functional sequences are well-known, and can be obtained or adapted from nearly any mammalian gene cloning vector that allows a cloned gene to be expressed at high levels by transfected mammalian cells. By making use of the pre-pro-

BDNF sequence that precedes the mature BDNF polypeptide, in place of the pre-pro-NGF sequence that precedes the mature NGF sequence, anterograde transport and release from the sensory neuron terminals within the BBB can be facilitated, in at least some types of neurons.

EXAMPLE 2: ADMINISTRATION OF ADENOVIRAL NGF VECTOR TO OLFACTORY EPITHELIUM, TO DELIVER NGF TO CHOLINERGIC NEURONS IN THE BASAL FOREBRAIN

Adenovirus-derived (or other virally-derived) vectors which carry a gene construct that encodes NGF (or some other CNS-active polypeptide) may be administered to the olfactory epithelium, of either a human patient (for medical purposes) or a test animal (for test or other research purposes), by using nasal instillation of an aqueous suspension of the vector, at a suitable titer concentration.

The aqueous carrier liquid should be compatible with adenovirus and olfactory epithelium vigor. Physiological saline (with buffering agents, if desired) can be used, and hypotonic or hypertonic solutions, or solutions containing any other component that may induce higher levels of viral transfection, can also be tested using routine experimentation. Articles such as Holtmaat et al 1996 provide information on dosage and administration techniques for efficient administration of adenoviral vectors via nasal instillation in mice; such procedures may be adapted for use in larger rodents such as rats or rabbits, or in other mammals, using methods known to those skilled in the art.

If desired, steps can be taken to increase (i) the extent and/or duration of contact between the fluid containing the gene vector and the olfactory neurons, and (ii) the receptivity of the neurons for taking in such genetic vectors. This can be done by, for example, administering a nasal decongestant to test animals (or human patients) a few hours prior to administration of the gene vectors, and by using an aqueous solution (and possibly a swabbing step, using a diluted solvent, such as isopropyl alcohol or acetone, that can help remove any mucous, oleaginous, or other viscous coating) to rinse and/or clean the nasal sinuses immediately prior to vector administration. The receptivity of the olfactory neurons may also be increased by using mild mechanical abrasion, using a small wire loop, rounded spatula tip, or similar tool. In general, neuronal projections in a surface area which has become irritated to a point of mild inflammation tend to be more receptive to cellular uptake of foreign molecules than cells which can be regarded as being in a quiescent or

resting state.

After allowing sufficient time for gene expression (usually in the range of about 24 to 72 hours), the effectiveness of gene vector delivery can be assessed by sacrificing some of the test animals, removing the olfactory epithelium, olfactory bulb, and basal forebrain, and processing each tissue type separately, to measure for the locations and concentrations of the vector-encoded polypeptide within that type of tissue.

The tasks of measuring and monitoring can be relatively simple where the vector-encoded polypeptide is distinguishably different from endogenous (presumably rodent) polypeptides. These types of analyses can use methods such as: (i) hybridization of cellular mRNA with DNA probes that are complementary to the vector mRNA sequences, but not to endogenous mRNA sequences, using procedures as described in articles such as Xian and Zhou 2000; (ii) techniques which use "polymerase chain reaction" (PCR) reagents and methods to detect DNA or mRNA sequences from the vector, as described in articles such as Chie et al 2000; and, (iii) immunostaining or similar methods which use monoclonal antibodies that selectively bind to the polypeptide or epitope tag encoded by the vector that was used, and do not bind to endogenous polypeptide in the test species (such antibodies are commercially available; they also can be prepared if desired, using methods disclosed in articles such as Conner 2000, Rush et al 2000, and Zhang et al 2000).

If desired, time-dependent levels of exogenous mRNA and/or polypeptide expression by transfected olfactory receptors can be measured by repeating one or more tests, over a range of times after administration of the genetic vector.

If desired, to provide control populations of cells and animals for purposes of data analysis, a genetic vector carrying a human (or epitope-tagged animal) polypeptide gene construct can be administered to the olfactory epithelium on one side of a test animal's nasal sinus, and a control vector which carries some other gene (such as a marker gene that encodes an easily-detected polypeptide) can be administered to the olfactory epithelium on the other side of the animal's nasal sinus. After the animal is sacrificed, histological and immunological examination of the left and right olfactory receptors, olfactory bulbs, and basal forebrain regions can be used to evaluate (i) polypeptide expression by the transfected olfactory receptors, and (ii) polypeptide transport and delivery by the BBB-straddling receptor cells to other classes of neurons located inside the BBB.

EXAMPLE 3: MONITORING NGF POLYPEPTIDE DELIVERY TO CHOLINERGIC

NEURONS IN THE BASAL FOREBRAIN

The quantity of NGF polypeptide (or any other vector-encoded CNS-active polypeptide) which is delivered via transfected olfactory receptor neurons (or any other type of sensory neurons which straddle the BBB and which are transfected by a genetic vector as disclosed herein), into CNS regions that are entirely within the BBB, can be monitored in any specific class or cluster of neurons of interest, using histological and immunological analysis of cells and tissues from sacrificed test animals.

Well-established immunological procedures, such as immunohistochemistry and "enzyme-linked immunosorbent assay" (ELISA) tests, can be used to monitor and quantify the production, release, and distribution of human NGF (or any other CNS-active polypeptide encoded by a genetic vector that has been administered to an animal), at any location of interest within the CNS, following administration of a genetic vector to olfactory receptor neurons, or other type of sensory neuron. If human NGF is the vector-encoded polypeptide, monoclonal and polyclonal antibody preparations which recognize and bind to human NGF, but not to mouse, rat, or other rodent NGF, are commercially available, or may be generated using procedures that are well documented in the literature (e.g., Conner 2000; Rush and Zhou 2000). Methods for measuring NGF levels using immunohistochemistry are described in articles such as Conner et al 1992, and methods for measuring NGF levels using ELISA are described in articles such as Zhang et al 2000, and in manufacturer's instructions which are included in NGF-ELISA detection kits that can be purchased from commercial sources.

As briefly mentioned above, monitoring of a vector-encoded human polypeptide, in lab animals such as mice or rats, can be rendered simpler and more certain if monoclonal antibodies are used that recognize and bind to the vector-expressed (presumably human) polypeptide, but which do not bind to the mouse or rat version of the same polypeptide. Hybridoma cell lines which express monoclonal antibodies that selectively bind to human NGF but not mice or rat NGF have been created, and such monoclonal antibody preparations are available. Similar hybridoma cell lines which express monoclonal antibodies that selectively bind to human but not rodent forms of other CNS-active polypeptides have also been created, or can be developed using methods known to those skilled in the art.

Alternately or additionally, as noted above, it is possible to incorporate an epitopic tag (such as c-myc) in a neurotrophic gene construct sequence, to facilitate immunological

detection of polypeptides expressed by that gene construct in transfected neurons, using methods described in articles such as Moller et al 1998. If such an approach is used, it is important to confirm that the polypeptide expressed by the vector construct is able to undergo all steps that are necessary for proper delivery to the neurons that are being targeted for treatment by that polypeptide.

For example, if the goal of a particular procedure is to deliver therapeutic polypeptides into the main cell bodies of basal forebrain cholinergic neurons, and an "epitopic tag" sequence is used to facilitate monitoring of the polypeptide, the targeted basal forebrain cholinergic neurons can be evaluated to ensure that they carried out uptake (such as receptor-mediated endocytosis) and retrograde transport of the vector-encoded, epitope-tagged polypeptides that were secreted by the transfected BBB-straddling neurons. These types of confirmatory tests can be carried out by using methods described in articles such as Altar & Bakhit 1991, Ferguson et al 1991, DiStephano et al 1992, and von Bartheld 2000, using reagents such as monoclonal antibodies that will bind specifically to the epitope tag sequence (and/or to a domain in the fusion polypeptide which contains part of the native amino acid sequence along with the tag sequence), to demonstrate that the vector-encoded polypeptides were taken up by the targeted basal forebrain cholinergic neurons.

If a vector-derived NGF carries an antigenic or epitopic tag, monoclonal antibodies which bind selectively to the tagged form may be creating using well-known methods, and can be used for any subsequent immunohistochemistry or ELISA measurements. If desired, confirmation that the delivery of NGF or some other CNS-active polypeptide (and its subsequent neurological and physiological effects on tissue inside the BBB) was indeed mediated by genetic vector administration to olfactory receptor neurons can be achieved by ablating these neurons in control animals, by treatment using a compound such as zinc sulfate solution, as described by Horowitz et al 1999.

Based on trans-synaptic tracer studies (e.g., Lafay et al 1991, Barnett et al 1993), it is generally anticipated that a vector-expressed polypeptide such as NGF will be detectable in the basal forebrain cholinergic neurons after allowing sufficient time for gene expression in the olfactory receptor neurons, which is likely to require a range of roughly 24 to 72 hours. This time period is anticipated to include anterograde transport of the polypeptide (after it has been expressed) to the receptor neuron's synaptic terminals in the olfactory glomeruli, secretion of the polypeptides within the glomeruli (roughly 8 to 24 hours), and transport of the polypeptides to and into basal forebrain cholinergic neurons (roughly 8 to

24 hours).

As discussed in Example 8, certain types of "trans-neuronal" vectors are also disclosed herein. That class of vectors may be able to be transmitted by sensory neurons to other types of neurons inside the BBB, in a manner which will transfect the recipient neuron with the foreign gene, causing one or more classes of "downstream" neurons lying wholly within the BBB to begin expressing the foreign therapeutic polypeptide.

However, unless specific steps are taken to provide "trans-neuronal" transport between neurons, it is assumed that: (i) transfection of initial or "primary" neurons (BBB-straddling neurons) by a genetic vector as disclosed herein will not lead to transmission of the vector to other non-primary neurons which are inside the BBB; and, (ii) transient gene expression will result, rather than permanent genetic transformation. If sensory neurons such as olfactory receptor neurons are used as the transfection targets, expression of the vector-borne gene(s) in such cells is expected to decrease substantially, and may stop entirely, during a period of several days to several weeks following the highest peak or plateau levels of polypeptide expression. If desired, reapplication of the same or a similar genetic vector to the olfactory receptor neurons can deliver an additional supply of NGF into BBB-protection CNS tissue.

EXAMPLE 4: MONITORING PHYSIOLOGICAL EFFECTS OF NGF DELIVERY TO CHOLINERGIC NEURONS IN THE BASAL FOREBRAIN

To enable and improve analysis of the actual physiological, behavioral, and other effects of CNS-active polypeptide delivery into BBB-protected CNS tissue using the genetic vector methods disclosed herein, experimental animals (or humans who have volunteered for clinical trials of this type of therapy) may be divided into two groups: (i) a test group which will receive a genetic vector which encodes NGF or some other selected neurotrophic or CNS-active polypeptide; and, (ii) a control group which receives a placebo treatment. The placebo treatment should use identical treatment of the instillation site (such as identical treatment with a decongestant, rinsing, swabbing, scraping, and/or other irritation of the nasal linings), followed by either: (a) nasal instillation of a plain saline solution with no genetic vector; or, (b) nasal instillation of a solution containing a genetic vector which might carry, for example, an innocuous and/or non-functional gene, a nonsense DNA sequence which does not encode any polypeptide, or a marker gene which encodes a polypeptide that can be easily detected if expressed in mammalian cells, but which has no

significant physiological effect.

When animals are tested, introducing a CNS-active polypeptide (such as NGF or some other neurotrophic factor) through the BBB and into protected brain tissue may lead to either or both of at least two categories of observable differences: (i) effects on the neuroanatomy of the brain, which can be evaluated by histological, immunological, or other biochemical analysis of sections of brain tissue removed from animals that have been sacrificed; and, (ii) observable and measurable effects on the behavior of the animal.

A number of tests have been developed for assessing what appears to be happening inside a laboratory animal's CNS system, based on observable and measurable forms of behavior (such as the ability of treated mice or rats to remember what they encountered in prior challenges involving mazes, water mazes, etc.). In addition, other such tests are being developed, and any such test which is currently known or hereafter discovered can be used, provided that it is appropriate for assessing the physiological effects of the polypeptide on targeted neurons lying wholly within the BBB.

It should be noted that many of these tests involve a surgical or drug intervention which inflicts some type of damage on the animal's CNS, to model an injury, stroke, neurodegenerative disease, or other CNS disorder. Subsequent tests then seek to evaluate whether a certain treatment can help such animals recover from the inflicted injury or disorder.

One example of such a test is the "fimbria fornix lesion model", a commonly used model in which the basal forebrain cholinergic neuron axons projecting to the hippocampus are "axotomized" (i.e., the main axon of a neuron is surgically severed; if untreated, this typically will cause the neuron to atrophy and die over a period of about two weeks or less, in most species). This induces atrophy and degeneration of the basal forebrain cholinergic neurons (e.g., Hefti 1994). However, that may not be a preferred injury model for evaluating the invention disclosed herein, because the basal forebrain cholinergic neurons which project through the fimbria fornix do not also project to the olfactory bulb.

Instead, preferred injury models for use herein should disrupt one of the neuronal pathways that is likely to be directly involved in, or affected by, the type of treatment disclosed herein. For example, surgically severing the cortical projections of neurons in the hindlimb of Broca (which project to both the olfactory bulb and the cortex) while leaving intact the olfactory bulb projection (so that retrograde axonal flow of NGF from the olfactory bulb is not blocked) would likely offer a better form of challenge to allow

researchers to evaluation the effects of the treatments disclosed herein.

An alternative animal model approach to severing an axonal tract is to ablate a selected type of appropriate target tissue, in a manner which will deprive basal forebrain cholinergic neurons of endogenous NGF, thereby inducing atrophy and degeneration of basal forebrain cholinergic neurons. This approach can generally model the effect of excitotoxic injury associated with stroke. Published examples include Sofroniew et al 1993, which describes ablation of hippocampal tissue. This procedure can be modified to ablate entorhinal cortex tissue, which receives innervation from the hind limb of Broca (Wenk et al 1980), to induce atrophy of basal forebrain cholinergic neurons in the hindlimb of Broca.

The general methods described in Sofroniew et al 1993 may be used to monitor the effect of NGF delivery (using genetic vectors as disclosed herein, such as by transfection of olfactory receptor neurons) on the basal forebrain cholinergic neuron cell body size, and/or by evaluating levels of one or more enzymes or other polypeptides that will be directly affected by the presence and quantity of NGF, which can therefore be used as an indicator polypeptide (one example of a candidate enzyme that might be useful for such measurements is choline acetyl transferase, or ChAT; this enzyme is required for synthesizing acetylcholine). Another neuroanatomical effect that can be observed and measured when NGF is administered into the CNS (such as by ventricular injection or infusion) to test animals which have had selected regions of cortical CNS tissue ablated, axotomized, or otherwise challenged, is shown by the ability of the NGF administration to prevent or reduce injury-induced reductions in the density of ChAT-immunoreactive fibers in the surviving cortex material (e.g., Garofalo et al 1992). Accordingly, methods described in articles such as Garofalo et al 1992 can be used to monitor the effects of NGF delivery (arising from genetic vectors as disclosed herein), on the density of ChAT-immunoreactive fibers in the surviving cortex, after a surgical or other intervention which ablates or otherwise damages one or more selected regions of cortical material.

In addition to neuroanatomical changes, cortical ablation results in reduction in certain measurable behaviors that can distinguish between normal and impaired performance on various tests that require learning and memory in test animals. Examples include the "Morris water maze", passive avoidance tests, responsive tests, and various other animal models, as described in articles such as Garofalo and Cuello 1994. It has been shown that administration of exogenous NGF into CNS tissue (such as by ventricular injection or infusion) in such test animals can significantly attenuate injury-induced deficits in behavioral

performance by such animals. Accordingly, such behavioral, memory, and/or learning tests may be adapted and used to monitor the effects of NGF delivery into the forebrain region via genetic vector treatment of neurons which straddle the BBB.

Aging also results in a reduction in memory and learning performance, as measured by tests such as the Morris water maze, (Fischer et al 1987 and 1991), and age-related declines in memory and learning performance have been used in efforts to measure and quantify, for example, the loss of memory and learning that occurs in Alzheimer's disease (both in human patients, and in various animal models of Alzheimer's disease). It has been shown that administration of exogenous NGF into CNS tissue (such as by ventricular injection or infusion) can significantly attenuate at least some types of age-related declines in memory and learning (Fischer et al 1987 and 1991). Accordingly, the types of learning and memory tests used in such animal models can be used to monitor the effects of NGF delivery into the CNS by the methods and genetic vectors disclosed herein.

The monitoring and measuring methods mentioned in Examples 3 and 4 herein are not exhaustive or exclusive; they can be supplemented by other monitoring and measuring methods known to those skilled in the art, or hereafter discovered.

EXAMPLE 5: CONSTRUCTION AND USE OF VECTORS DERIVED FROM HERPES SIMPLEX VIRUS FOR NON-INVASIVE DELIVERY OF NGF

The Examples above describe the use and evaluation of adenovirus-derived vectors, to genetically transfect BBB-straddling olfactory receptor neurons, as a route and method for non-invasive delivery of NGF into BBB-protected CNS tissue. However, it should be recognized that adenoviral vectors are not the only types of genetic vectors that can be used for non-invasive delivery of polypeptides into BBB-protected CNS tissue. Accordingly, Examples 5-8 describe the use of several other classes of genetic vectors.

To construct genetic vectors which are derived from herpes viruses, and which carry one or more "passenger" genes which encode CNS-active polypeptides as disclosed herein, methods described in articles such as Goins et al 1999 or Federoff et al 1992 can be used. HSV-derived vectors are capable of transfecting a wide variety of human cells, including olfactory receptor neurons, and they can induce transfected BBB-straddling neurons to express passenger genes, and to secrete significant levels of such polypeptides.

Accordingly, HSV-derived vectors may be used, if desired, in a manner directly comparable to the use of adenovirus-derived vectors as described in Examples 1-4. The

methods described in Examples 2-4 (supplemented when appropriate by other or additional methods known to those skilled in the art) may be used for nasal instillation of HSV-derived vectors, and for post-transfection monitoring and evaluation.

EXAMPLE 6: CONSTRUCTION AND USE OF LIPID-BASED VECTORS FOR NON-INVASIVE DELIVERY OF NGF

Methods for preparing DNA plasmid-lipid complexes designed for transfecting mammalian cells are described in numerous publications, such as the chapter by Nabel (pp. 127-133) in *Methods in Molecular Medicine: Gene Therapy Protocols* (P. Robbins, ed., 1997). A range of liposome preparation kits designed for use in gene transfer are available commercially, or can be designed and created by any skilled technician using published methods. The preferred choice of lipid formulation and preparation parameters (including lipid and DNA concentrations and ratios in the preparation mixture, temperature, etc.) for use with a particular size, type, or concentration of plasmid DNA or other DNA preparation can be determined by routine testing of various preparative mixtures, using parameter ranges and confirmatory tests that are discussed in numerous articles and in the instructions that accompany most commercially available kits.

Administration of lipid-based genetic vectors to olfactory epithelium, via nasal instillation of a lipid-gene complex suspended in an aqueous saline solution or other carrier liquid, can use the same general procedures described in Example 2, adapted for such use by means known to those skilled in the art. The methods described in Examples 3 and 4 (supplemented when appropriate by other or additional methods known to those skilled in the art) may be used for post-transfection monitoring and evaluation.

EXAMPLE 7: CONSTRUCTION AND USE OF DNA VECTORS THAT TARGET ENDOCYTOTIC RECEPTORS; CYCLIC LIGAND SELECTION PROCESS

The general principals and procedures that can be used to prepare "receptor-targeting gene vectors" are described in various publications, such as the chapter by Findeis (pp. 135-152) in *Methods in Molecular Medicine: Gene Therapy Protocols* (P. Robbins, ed., 1997). As described previously, these vectors contain ligands that will bind to certain types of receptors that are exposed on the surfaces of neurons.

As mentioned in the Background section, a number of known types of neuronal surface receptors undergo a process called "endocytosis", after a ligand molecule becomes

bound to the receptor. As suggested by the name, an endocytotic receptor will be taken inside the cell, after its ligand molecule becomes bound to it. This type of activity can be shown by tests using radiolabelled ligands.

One example of an endocytotic receptor is the "p75" receptor, which is accessible on the surfaces of various types of sensory neurons (including olfactory receptor neurons). It has been shown to exist in both humans and rats, making it highly useful for various types of research. Also known as the p75NGFR receptor (where NGFR indicates "nerve growth factor receptor"), it is of special interest for use as described herein, because it has been shown to be "up-regulated" (i.e., the expression of mRNA encoding the receptor is increased, and the number of receptors that appear on the surfaces of the neurons is increased) in various types of neurons that are subjected to crisis or stress conditions. As examples, p75 expression increased in the motor neurons of rats following a peripheral nerve injury (Yan et al 1988), and p75 expression also increased in the motor neurons of human patients suffering amyotrophic lateral sclerosis (Seeburger et al 1993).

Selection and/or creation of an appropriate ligand that will bind to an endocytotic receptor on the type of neuron being targeted (ideally, in the species being tested or treated) is key to construction of an effective receptor-targeting gene vector of this type. A variety of such ligands are already known, and others can be created, using methods such as briefly summarized above.

Since endocytotic receptors are proteins, it is usually possible to create a complementary polypeptide that will bind to any such receptor, by using a polypeptide sequence from the receptor as an antigen, during the creation of monoclonal antibodies. Using well-known techniques, the antigenic sequence derived from the receptor is injected into animals such as mice, rats, or rabbits; the resulting antibody-producing cells are then fused with an immortalized cell line; and, the resulting "hybridoma" cells are screened, to identify and isolate a clonal cell line which secretes monoclonal antibodies that will bind with high affinity to the receptor of interest. Once the desired monoclonal antibody line has been created and identified, a smaller domain or fragment usually can be isolated from the variable binding portion of that monoclonal antibody (often referred to by acronyms such as the "scFv" fragment, where "sc" refers to "single chain" and "Fv" refers to the variable fragment, which comprises the binding domain). A gene sequence which encodes that binding fragment can then be incorporated into a plasmid or other vector, to allow unlimited quantities of the receptor ligand fragment to be synthesized by fermentation of

host cells.

Using that general type of approach, monoclonal antibodies can be created which will bind to essentially any type of known endocytotic receptor, on any type of sensory neuron having one or more peripheral projections.

To continue the example of the p75 receptor, a monoclonal antibody known as 192-IgG (described in Chandler et al 1984), has been shown to bind with high affinity to p75 receptors in rats. The 192-IgG monoclonal antibody (as well as various fragments derived from it) has been shown to undergo endocytosis, and retrograde transport, in peripherally-projecting neurons which express the p75 receptor (Yan et al 1988).

Once a polypeptide sequence that can serve as a receptor-binding ligand has been identified, various reagents can be used to temporarily couple a DNA segment to the ligand polypeptide, thereby creating a polypeptide-DNA complex. In one preferred method, a polypeptide can be created having "polylysine" domain, with a series of lysine residues (or other amino acid residues having a positive charge at physiological pH) coupled to the ligand polypeptide. This can be done by any of several methods, such as adding a string of lysine codons to the polypeptide-encoding gene which is used to express the polypeptide in a host cell, or chemically conjugated polylysine to a polypeptide by using bifunctional crosslinking reagents. Because of chemical attraction (a polylysine strand has a positive charge, and a DNA strand has a negative charge), DNA segments will adhere in a non-covalent manner to polypeptides that have polylysine "tails". When the ligand portion of the polypeptide binds to an endocytotic receptor, it commences a process that will draw the entire polypeptide-polylysine-DNA complex (plus the receptor protein as well) into the cell. After that complex has been taken inside the cell, the DNA molecule will be released, and can be retrogradely transported inside the cell.

Alternately, a normal ligand for a neurotrophin receptor (such as NGF, which binds to NGF receptors) may be used. Radiolabelled NGF has been shown to undergo receptor-mediated internalization and retrograde transport, in sensory neurons which express a receptor called "trkA" (e.g., Barbacid 1995); accordingly, the NGF polypeptide itself can be used as a ligand, for cells having that receptor. It should be noted that the NGF polypeptide itself (and, it is believed, various other neuronal receptor ligands) has a net positive charge, similar to the positive charge found on histones, a class of DNA-binding proteins that are associated with chromosomes; accordingly, the need to first attach a polylysine or other similar positively-charged domain or conjugate to an NGF or other

positively-charged polypeptide might be avoided entirely, or minimized, and the polypeptide itself may be able to function as both a receptor targeting ligand and a DNA carrier.

Various other known compounds also provide good candidates which can be evaluated for potential use as ligands which can bind to endocytotic receptors on one or more types of BBB-straddling neurons, and/or as peptide sequences which can promote retrograde transport or some other function after a genetic vector or some portion thereof has been taken inside a neuron. As just one example, Wiley and Lappi 1993 describes a conjugate formed by coupling (i) monoclonal antibody 192-IgG, which binds to the p75 neuronal receptor, to (ii) a plant-derived toxin called saporin, which is believed to promote intracellular transport to a neuronal cell body, where it inactivates ribosomes. This conjugate was reported by Wiley and Lappi to be useful for causing selective and targeted neuronal lesions, for research purposes; accordingly, it can be regarded herein as a "probe" compound. It may be possible, using methods known to those skilled in the art, to modify and adapt that or similar types of probe compounds, to render them nonlethal to neurons, in a manner that will allow a segment of DNA to be (i) non-covalently coupled to the "probe" compound; (ii) transported to the neuronal cell body, with the aid of the probe compound, and then (iii) released from the probe compound, into the cell body, after the internalization and transport steps have been completed.

In addition, numerous types of neurotoxins have been derived from the venoms of spiders, wasps, snakes, marine snails, and other venomous organisms. One of the more common features of neurotoxins is that they bind (often with extremely high levels of binding affinity) to one or more types of receptors and/or ion channels on the surfaces of neuronal projections; this is one of the principle mechanisms that organisms in the wild use to paralyze or otherwise incapacitate their prey, and to defend against attackers. A substantial number of such neurotoxins have been modified by research biochemists, to form analogs, conjugates, and other variants and derivatives which have lower, non-toxic levels of binding affinity for neuronal receptors or ion channels, in the search for therapeutic agents that can be medically useful without inflicting pain at the levels normally caused by a bite or sting from a highly venomous animal.

In addition, various types of toxins from pathogenic microbes (including tetanus toxin, cholera toxin, etc.), and various compounds derived from plants (including compounds that fall within a category known as lectins or agglutinins) offer a number of candidates for use as described herein, which can be evaluated to determine whether that

can provide selective ligands that will help a genetic vector bind to and transfect one or more types of BBB-straddling neurons.

Accordingly, any such known or hereafter-discovered neurotoxin, microbial toxin, plant lectin, or other functionally similar compound which can bind selectively to one or more receptors, ion channels, proteins, glycoconjugates, or other molecules that are found on the surfaces of BBB-straddling neurons, and any analog, conjugate, or other variant or derivative of any such neuron-binding compound, can be evaluated for potential use as disclosed herein, as a ligand that may help genetic vectors selectively bind to and transfect one or more types of BBB-straddling neurons.

As a third alternative approach, a "combinatorial chemistry" approach which uses phage display libraries can be used, as described in the Preferred Embodiments section, and as illustrated in FIG. 7. Briefly, this approach uses phage display libraries to generate an array of potential ligand polypeptides, wherein each phage expresses a single polypeptide fragment. The entire array of phages is screened, using a process of receptor-mediated endocytosis carried out by neurons *in vivo*, such as in rats that have had ligature-type constrictions placed on nerve bundles such as a sciatic nerve. Intact phage particles (which can infect host bacteria) are subsequently harvested from a segment of extracted nerve cells, immediately adjacent and "distal" to the point where the ligature was placed. The harvested phage particles are then inoculated into *E. coli* cells, which are used to grow a subsequent generation of phages. This new generation of phages will contain those particular phages which were present inside the nerve cells, at the location adjacent to the ligature. As described in more detail in the Background section, because of how these phages were inoculated into and subsequently harvested from a treated lab animal, the particular phages which are selected by this screening process presumably must express ligand polypeptide sequences which caused the selected phages to be: (i) taken inside the peripheral projections of neurons, by a process of endocytosis; and, (ii) retrogradely transported, inside the neuronal axon, to the site adjacent to the ligature.

By repeating that type of screening and selection process several times (and by subjecting phage lines which performed well to random or targeted mutagenesis, if desired), clonal phage lines can be identified which will have incorporated genes that encode polypeptide sequences which are highly effective as ligands that can bind to endocytotic receptors and trigger endocytosis. Those phages can be analyzed, and the gene and amino acid sequences of those ligands can be determined, for subsequent use in creating highly

effective receptor-targeting transfection vectors.

If desired, this type of cyclic screening process can be further enhanced by the use of various types of assays, such as "fluorescent activated cell sorting" (FACS), which can be carried out using known types of machines that are usually called "flow cytometers". As an example of how this process can be used, plasmid, phage, or "phagemid" DNA can be fluorescently labelled, using a compound such as rhodamine. Cells grown in tissue culture, which are known to have p75 receptors on their surfaces, are incubated with various polypeptide-DNA complexes, and the cells are then passed through a cell sorting machine equipped with a fluorescent detector. If the fluorescent light emitted by a particular cell is relatively strong, that will indicate that a certain cell took in large quantities of labelled polypeptide-DNA complexes, and a synchronized very brief jet of gas or fluid can cause the fluid stream carrying that particular cell to be diverted into a separate collection device. In this manner, cells which contain large quantities of "uptaken" DNA can be isolated rapidly and easily, using high-speed automated equipment, and can subsequently be reproduced, analyzed. or otherwise processed to evaluate or replicate the types of polypeptides which caused high levels of polypeptide-DNA uptake into the cells. This is just one example of how automated analysis and manipulation can be used to simplify and enhance this invention, and other methods will also become apparent to those skilled in the art.

As yet another alternative, DNA-protein complexes may be prepared by coupling a DNA segment to a known type of capsid protein derived from adenovirus (or from various other viruses which act in a similar manner). This adenoviral capsid protein is known to help promote efficient release of the vector-carried DNA from the endocytotic vesicle, after the complex has entered the target cell. This approach is described in the chapter by Curiel (pp. 25-40) in *Methods in Molecular Medicine: Gene Therapy Protocols* (P. Robbins, ed., 1997). If desired, the adenoviral capsid protein sequence and the receptor-binding ligand sequence may be combined, in a fusion protein.

Nasal instillation of these types of receptor-targeting transfection vectors can use the same general procedures described in Examples 2 and 6; those general procedures can be adapted specifically for use with receptor-targeting ligand vectors, by means known to those skilled in the art. The methods described in Examples 3 and 4, and other methods known to those skilled in the art, can be used for post-transfection monitoring and evaluation.

EXAMPLE 8: CONSTRUCTION AND USE OF TRANS-NEURONAL VECTORS TO

DELIVER NGF GENE TO SECOND-ORDER NEURONS IN THE BRAIN

This example describes a method of developing "transneuronal" gene vectors, which may be able to transport foreign genes (rather than just polypeptides expressed by foreign genes) into certain classes of neurons that are located entirely within the BBB. In other words, the goal of such transneuronal gene vectors is to transfect, and genetically transform, not just neurons which straddle the BBB, but also other "secondary" or "second order" neurons which share synaptic junctions with transfected BBB-straddling "primary" neurons (and, potentially, tertiary neurons as well, which share synaptic junctions with secondary neurons). Transneuronal vectors may be able to greatly increase both the quantity and the distribution of new and/or supplementary polypeptides, which can be secreted by neurons that reside wholly within the BBB. Such treatment may become an effective way to treat certain neurodegenerative diseases characterized by widespread and disseminated brain damage, such as Parkinson's disease and Alzheimer's disease.

It should be recognized that these types of transneuronal vectors are not expected to replicate and produce multiple copies of themselves, after they enter secondary (or tertiary, or subsequent) neurons. Instead, the goal of such transneuronal vectors is simply to place these vectors in "downstream" neurons which are fully within the BBB, rather than limiting their placement to "primary" neurons which straddle the BBB. However, it is recognized that in some cases (which will become more probable and more frequent if additional steps are taken to promote integration of the gene sequences into chromosomes within the downstream neurons, by steps such as making use of terminal repeats derived from adeno-associated virus to bracket the transcription unit of the plasmid), this approach may lead to non-invasive methods of genetic therapy on CNS neurons that are entirely within, and protected by, the blood-brain barrier.

The currently anticipated approach to constructing transneuronal vectors arises out of various facts known about transneuronal transport of certain pathogens and polypeptides, including: (i) the "nontoxic fragment C" of tetanus toxin (e.g., Knight et al 1999); (ii) barley lectin (Horowitz et al 1999); and, (iii) wheat germ agglutinin (Yoshihara et al 1999). Barley lectin and wheat germ agglutinin have been shown to undergo active transneuronal transport from olfactory receptor neurons, into basal forebrain cholinergic neurons, and into other classes of neurons as well, including neurons in the locus ceroleus and raphe nuclei. Other polypeptide sequences with transneuronal transport capability can also be discovered and created, using the same type of combinatorial selection strategy described in Example

7, above; this can be done by repeated cyclic testing of phage display libraries, to identify phages which have gene sequences that encode polypeptides which drive transneuronal transport of phage into secondary (or tertiary, or subsequent) neurons.

Known methods can be used to construct a gene vector using a polypeptide which is known to have transneuronal transport capability. For example, polylysine can be covalently bonded to a transport polypeptide, using a chemical reaction, or using a genetically modified gene which encodes for numerous lysine (or other positively charged) residues at one end of the polypeptide. Since polylysine is positively charged, it will attract DNA segments (which are negatively charged) when the polypeptide is mixed with DNA segments in solution. This leads to moderately strong but noncovalent binding of the DNA to the polypeptide. This type of preparation is described in Knight et al 1999.

Administration of these genetic vectors to olfactory epithelium, via nasal instillation of vectors suspended in an aqueous saline solution or other carrier liquid, can use the same general procedures described in Example 2, adapted for such use by means known to those skilled in the art.

The methods described in Examples 3 and 4 (supplemented when appropriate by other or additional methods known to those skilled in the art) may be used for post-transfection monitoring and evaluation. Demonstration of expression of transneuronally transported NGF gene constructs within neurons inside the BBB (such as basal forebrain cholinergic neurons) can be done with sensitive procedures such as *in situ* hybridization and/or polymerase chain reaction (PCR). As described in Example 3, detection of expression will be facilitated by making use of NGF gene sequences which express polypeptides that are distinguishably different from the corresponding polypeptides in the host species.

Additional monitoring of the physiological and behavioral effects caused by such transneuronal vectors can be done by procedures such as described in Example 4.

EXAMPLE 9: CONSTRUCTION AND USE OF VIRAL VECTORS TO DELIVER ANTI-NGF POLYPEPTIDES INTO DORSAL HORN REGIONS IN THE SPINAL CORD

While Examples 1-8, above, describe delivery of neurotrophic polypeptides such as nerve growth factor (NGF) to help stimulate neuronal growth or repair, this Example 9 and several following examples describe a completely different type of polypeptide, referred to

herein as an "anti-NGF" polypeptide. Such polypeptides, if they are transported through the BBB and delivered into properly targeted locations, can help treat and reduce various conditions, such as neuropathic pain or autonomic dysreflexia. Such polypeptides can work by at least two known mechanisms: (i) by binding to NGF molecules in cerebrospinal fluid (CSF) or synaptic fluid, thereby inactivating the NGF molecules by rendering them unable to bind to NGF receptors; and/or, (ii) by binding to NGF receptor proteins on neurons, thereby occupying those receptors and rendering them inaccessible to NGF molecules, in a manner which does not trigger the cellular reactions that occur when free NGF molecules bind to the receptors.

Anti-NGF polypeptides which bind to free NGF molecules in CSF or synaptic fluid are known, and are described in articles such as Ruberti et al 1993 and 1994. Gene sequences which encode these anti-NGF polypeptides are also known, and can be incorporated into complete gene constructs having suitable gene promoters which will drive gene expression in mammalian cells, as disclosed in Example 1 above.

If such a gene construct is intended for expression in a nocioceptive neuron, rather than an olfactory receptor or other type of sensory neuron, attention must be given to the gene promoter that will be used to drive expression of the gene. A range of potential gene promoters that function in nocioceptive neurons are known, and suitable promoters which can drive desired levels of gene expression in nocioceptive neurons can be selected from those candidate promoters by routine experimentation. Preferred promoter selection also can be influenced by expression levels that occur naturally in different types of neurons.

For example, by using a gene promoter which drives expression of the CGRP gene (described in Watson et al 1995), expression of a vector-carried anti-NGF gene can be restricted to transfected nocioceptive neurons which express the neuropeptide CGRP. The CGRP promoter may be especially useful if one objective of a treatment is to reduce the level of NGF in the spinal cord of a patient suffering from neuropathic or other chronic pain, because the activity of this promoter is enhanced in the presence of NGF. Thus, when NGF levels in the spinal cord increase, this promoter may be able to increase synthesis and secretion of anti-NGF in response, thereby creating a self-limiting, self-regulating gene expression system.

A gene construct having an anti-NGF coding sequence and a selected promoter can be placed in a gene vector derived from replication-deficient adenoviruses, using methods such as described and cited in Example 1 and Ruberti et al 1993 and 1994, or in a gene

vector derived from replication-deficient herpes viruses, using methods such as described in Example 5 and the articles cited therein.

Since this example relates to methods of reducing and controlling NGF-aggravated neuropathic pain, the anti-NGF vectors disclosed herein will not be inserted into sensory receptor neurons such as olfactory neurons. Instead, these vectors will be injected into regions that contain high concentrations of nocioceptive nerves, including subcutaneous tissues or muscle tissue in regions that are (i) plagued by neuropathic pain, such as allodynia, and/or (ii) innervated by BBB-straddling nocioceptive neurons, using methods such as described by Sahenk et al 1993, or as determined to be optimally effective during clinical trials or during a treatment procedure involving a specific patient.

After injection into regions with nocioceptive nerve projections, at least some of the anti-NGF gene vectors will contact nocioceptive neuron projections outside the BBB. Using the same mechanisms that viruses normally use to inject DNA into cells during infection, the viral vectors (or analogous mechanisms used by non-viral vectors, as described in Examples 6-8) will inject the genetically-engineered vector DNA into the outer projections of the BBB-straddling nocioceptive neurons that innervate those tissues. Using retrograde transport, the vector DNA will be carried through the cell cytoplasm into neuronal regions called dorsal root ganglions, which flank the spinal cord. The gene which encodes the anti-NGF polypeptide will be expressed in the nocioceptive neurons residing within the ganglions (the plural form is also spelled "ganglia"), thereby creating anti-NGF polypeptide molecules within BBB-straddling neurons.

At least some of these anti-NGF polypeptides will be transported, using normal anterograde transport, through the axons of BBB-straddling neurons, until they reach synaptic terminals or similar exit points which are located in "dorsal horn" regions (and possibly other regions) of the spinal cord, in tissue that is enclosed within and protected by the BBB. The efficiency of this anterograde transport step may be enhanced by including a leader or signal peptide, at the start of the secreted or mature polypeptide, that will instruct the transfected cell to package the polypeptide in vesicles containing neurotransmitter peptides destined for anterograde transport and release at synapses lying within the BBB. An example of such a leader sequence suitable for use in nocioceptive neurons is the pre-pro-BDNF sequence, which directs nocioceptive neurons to anterogradely transport mature BDNF (which these neurons normally synthesize) into dorsal horn regions within the spinal cord.

When released by nocioceptive neuron terminals at these locations, the anti-NGF molecules will bind to NGF molecules, thereby reducing the amount of free NGF in the spinal tissue at that location. Reduced access to NGF does not kill mature nocioceptive neurons; however, it does lead to a reversible form of suppression (often called "down regulation") of nocioceptive functions and activity, as demonstrated by direct administration of anti-NGF into laboratory animals, as described in articles such as Christensen et al 1996.

Anti-NGF may be detected in the spinal cord after allowing sufficient time for gene expression in the dorsal root ganglion (anticipated to be about 24 to 72 hours) and anterograde transport and release of the anti-NGF into the dorsal horn regions of the spinal cord (anticipated to be another 8 to 24 hours).

In laboratory animals that have been sacrificed, the release of anti-NGF polypeptides in the spinal cord can be monitored directly with appropriate methods, such as electron microscopy, immunological staining, and various methods of labelling the vector-derived anti-NGF, such as inclusion of an appropriate antigenic tag in the amino acid sequence of the polypeptides encoded by the vector gene. It also may be possible to monitor the concentration and/or effects of anti-NGF by measuring the concentration of free NGF. When anti-NGF is released, it will bind to and neutralize endogenous NGF in the spinal cord. Accordingly, changes in the level of NGF in the spinal cord can be monitored by making use of various immunological procedures, such as those outlined in Example 3, and applying these procedures to examination of the spinal cord tissue receiving innervation from the transfected sensory neurons. Changes in the level of NGF in the spinal cord can also be inferred from characteristic neuroanatomical and behavioral changes, as outlined in more detail in Example 10.

EXAMPLE 10: MONITORING PHYSIOLOGICAL AND BEHAVIORAL EFFECTS OF ANTI-NGF DELIVERY INTO DORSAL HORN REGIONS IN THE SPINAL CORD

To allow for statistical comparison of test results, experimental animals may be divided into two groups. One group (the test animals) will receive the anti-NGF gene vector, as described above, while the other group (the control group) will receive a vector containing noncoding DNA, an innocuous gene, or a marker gene that has no known neuroactive effect on CNS tissue. The physiological effects of administering anti-NGF into the spinal cord include effects on the neuroanatomy of the brain and effects on the behavior

of the animal, as discussed in articles such as Christensen et al 1996 and Christensen et al 1997.

Appropriate choice of animal model(s) is important in evaluating the physiological effects of anti-NGF delivery into the spinal cord. In addition to animal models of inflammatory pain, various models of hyperalgesia and "allodynia" (chronic and/or neuropathic pain, in which moderate stimuli are interpreted by the CNS as severe pain) can be generated by injuring or challenging a peripheral nerve.

As just one example of a common method for modelling neuropathic pain, and for testing potential treatments for neuropathic pain, a loop ("ligature") of suture thread can be surgically placed and then tightened around half to two-thirds of the sciatic nerve, in one of the hind legs of an animal such as a rat. Within several days to a week, the leg will become hyper-sensitized and susceptible to serious pain in response to mild stimulus. Rats that have been treated in that manner can then be placed in a special box with automated sensors, which can measure and record how many seconds pass after the floor plate begins to warm up, until the rat lifts the hyper-sensitive paw to get it off the warmed surface. If a candidate treatment can extend the average number of seconds that pass before nerve-ligated paws are raised, until those values approach the comparable times for unoperated control animals, that indicates that the treatment may be effective in reducing neuropathic pain, and may deserve more elaborate testing on larger animals, and/or clinical trials on human patients.

It should be recognized, however, that peripheral nerve ligation or other challenges or injuries may interfere with retrograde transport of vector DNA from an injection site to the dorsal root ganglion, and may complicate the design and interpretation of such tests for use to evaluate the therapies disclosed herein. Accordingly, challenges which directly affect and involve the spinal cord may be preferred in at least some situations. Chronic or neuropathic pain due to spinal injury is frequently observed in clinics, and over-production of NGF within the spinal cord has been implicated in a condition known as "primary afferent sprouting", which often follows spinal cord injury (e.g., Krenz et al 2000). Accordingly, spinal cord injury models which reproducibly causes hyperalgesia or chronic pain can be used to monitor the effects of anti-NGF delivery into the spinal cord on hyperalgesia or chronic pain. Examples include the spinal cord injury model described in Krenz et al 1999, which is believed to be suitable for evaluating the physiological effects of anti-NGF delivery into the spinal cord using the methods disclosed herein.

Neuroanatomical changes in the pattern of sensory innervation of the dorsal horn

associated with hyperalgesia or chronic pain includes abnormal sprouting of "A" fibers and nocioceptive CGRP-containing fibers into lamina II of the dorsal horn (e.g., Bennet et al 1996). The effect of administration of an anti-NGF gene vector on such neuroanatomical changes can be monitored by using the methods described by Bennet et al 1996 or Christensen et al 1997, while using an agent such as cholera toxin B subunit to transganglionically label the A fibers, and by using CGRP immunohistochemistry to visualize the nocioceptive (NGF-responsive) fibers in the dorsal horn.

Alternately, the effect of delivery of anti-NGF on the number and extent of synaptic connections between peripherally-projecting sensory neurons and second-order neurons in the spinal cord may be evaluated by transneuronal tracing methods, using procedures such as described in Blessing et al 1994.

Established methods for functional monitoring of pain responses in animal models include: (i) measuring foot withdrawal latency in response to thermal stimulus, as briefly summarized above and described in more detail in articles such as Romero et al 2000; (i) measuring responses to application of von Frey hair stimulus, as described in articles such as Deng et al 2000.

Another condition that may be susceptible to treatment by anti-NGF or comparable polypeptides is often referred to as "autonomic dysreflexia". This condition occurs most commonly in patients with spinal cord injuries in the upper thoracic or cervical region, which disrupt the normal set of connections in the preganglionic sympathetic neurons in the upper spinal cord. As a result, the normal feedback control mechanisms in the autonomic nervous system can be disrupted, in ways that can cause a normal stimulus (such as colon distension, indicating full bowels) to trigger other reflex results, some of which can be potentially life-threatening (such as increases in heart rate and blood pressure, to levels which can pose major risks of severe cardiovascular consequences, such as a heart attack or stroke).

As shown in animal model studies (e.g., Krenz et al 1999), direct infusion of anti-NGF into the spinal cord in animal models of this condition can prevent or help control this type of unwanted reflex response, by helping suppress abnormal and unwanted innervation of the dorsal horn by nocioceptive neurons, as can occur after that type of spinal cord injury.

Therefore, the use of this invention to introduce anti-NGF polypeptides into spinal regions that are protected by the BBB, by using genetic vectors to transfect exposed and

accessible projections of nocioceptive neurons (and possibly other spinal neurons, including motor neurons), holds substantial promise in treating autonomic dysreflexia.

EXAMPLE 11: CONSTRUCTION AND USE OF LIPID-BASED GENE VECTORS TO DELIVER ANTI-NGF POLYPEPTIDES INTO DORSAL HORN REGIONS IN THE SPINAL CORD

The procedures described in Example 9 and articles cited therein may be used to create an anti-NGF gene construct that will be expressed in one or more targeted classes of nocioceptive neurons. The resulting gene construct copies can be placed inside a DNA vector such as a plasmid or other stable form, using known methods. The DNA vectors can then be placed inside lipid vesicles (liposomes), using methods such as described in Example 6 and in the chapter by Nabel (pp. 127-133) in *Methods in Molecular Medicine: Gene Therapy Protocols* (P. Robbins ed., 1997).

The resulting liposome vectors may be administered to peripheral projections of nocioceptive neurons by means such as injecting an aqueous suspension of liposomes in saline solution into regions that contain high concentrations of nocioceptive nerves, including subcutaneous tissues or muscle tissue in regions suffering from neuropathic or other chronic pain, using methods such as described by Sahenk et al 1993, or as determined to be optimally effective during clinical trials or during a treatment procedure involving a specific patient. At least some of the liposome vectors will adhere to and merge with nocioceptive neuron projections which are located outside the BBB, in the muscle tissue, and that reaction will deliver the anti-NGF encoding DNA which was carried by the liposomes into the neuronal projections. Retrograde transport of the DNA segments will carry at least some of the anti-NGF DNA to the neuronal cell body, where the anti-NGF genes will be expressed into anti-NGF polypeptides.

Subsequent delivery of the anti-NGF polypeptides into the spinal cord, and the physiological and behavioral effects of the anti-NGF polypeptides in spinal tissue, can be measured and monitored using procedures such as described in Examples 9 and 10, above.

EXAMPLE 12: CONSTRUCTION AND USE OF DNA VECTORS THAT TARGET ENDOCYTOTIC RECEPTORS ON NOCIOCEPTIVE NEURONS, TO DELIVER ANTI-NGF POLYPEPTIDES INTO DORSAL HORN REGIONS IN THE SPINAL CORD

Various known methods, such as those described in Example 9, can be used to create anti-NGF gene constructs that will be expressed in nocioceptive neurons. Other known methods, such as those described in Example 11, can be used to place the anti-NGF gene constructs into plasmid form or other stable forms that can be transported into nocioceptive neurons by non-viral vectors such as liposomes.

These stable DNA forms can then be used to form protein-DNA complexes, which incorporate polypeptide segments that will bind in a specific manner, as ligands, to nocioceptive neuron receptors which undergo endocytosis, using procedures such as described in Example 7. Various such receptor-specific polypeptide segments are already known, and others can be identified and developed using the phage library approach described in Example 7.

Accordingly, these steps, when compiled together in proper sequence, will create genetic vectors that can specifically target endocytotic receptors on peripheral projections of nocioceptive neurons, in regions that are not enclosed within the BBB and which therefore provide relatively simple access to the peripheral projections. Such receptor-specific endocytotic gene vectors can be used to transfect such neurons with anti-NGF genes that will be expressed in those neurons, and the neurons themselves will then deliver and secrete the anti-NGF polypeptides in dorsal horn regions of the spinal cord, in a manner which can help control and reduce neuropathic pain and possibly other pain disorders.

Delivery of such anti-NGF polypeptides into spinal cord tissue can be monitored using procedures such as described in Example 9, and the physiological and behavioral effects of anti-NGF polypeptides in such spinal cord tissue can be evaluated using procedures such as described in Example 10.

EXAMPLE 13: TRANS-NEURONAL ANTI-NGF VECTORS THAT WILL TRANSPORT ANTI-NGF GENE TO SECOND-ORDER NEURONS IN THE SPINAL CORD

Gene constructs (including suitable gene promoters) that can express anti-NGF polypeptides in nocioceptive neurons can be created as described in Example 9. These gene constructs can be placed in plasmids or other stable forms, using known methods.

Such anti-NGF plasmids or other DNA vectors can then be coupled to "transneuronal polypeptides" that can help enable and promote the transfer of a protein-DNA complex from one neuron, to another. Various such "transneuronal polypeptides" are

known, and include, for example, the "nontoxic fragment C" of tetanus toxin (e.g., Knight et al 1999), barley lectin (Horowitz et al 1999), wheat germ agglutinin (Yoshihara et al 1999) (also listed in Example 8, above).

By binding reversibly to proteins that are (i) exposed at synaptic terminals in the periphery, and (ii) internally transported by the BBB-straddling neuron to synapses within the BBB, such "transneuronal polypeptides" can enable the transport of protein-DNA complexes through the cytoplasm of transfected "primary" BBB-straddling nocioceptive neurons, through the BBB, and into neuronal terminals located in the dorsal horn, and possibly elsewhere in spinal tissue. The transneuronal proteins will then help the protein-DNA complexes exit the synaptic terminals of the BBB-straddling nocioceptive neurons, and enter into adjacent spinal cord neurons that are located entirely within the BBB, thereby transfecting "secondary" spinal cord neurons protected by the BBB.

Delivery of such anti-NGF gene vectors and encoding sequences into BBB-protected "secondary" spinal neurons can be monitored by methods such as *in situ* DNA probe hybridization and PCR analysis, using spinal cord cells and tissue from sacrificed lab animals. Expression of anti-NGF polypeptides by transfected "secondary" spinal neurons can be monitored using procedures such as described in Example 9, and the physiological and behavioral effects of anti-NGF genes and polypeptides in such spinal cord cells can be evaluated using procedures such as described in Example 10.

EXAMPLE 14: USE OF ADENOVIRAL VECTORS FOR TRANSFECTING SPINAL MOTOR NEURONS THAT WILL TRANSPORT NEUROTROPHIC POLYPEPTIDES TO UPPER MOTOR NEURONS

Procedures for preparing non-pathogenic adenoviral vectors that cannot replicate in normal cells have been published in articles such as Graham and Prevec 1995. Articles that describe examples of such vectors which contain genes that encode various neurotrophic factor include Dijkhuizen et al 1997, Gravel et al 1997, Baumgartner et al 1998, and Romero et al 2000.

Gene sequences which encode "glial cell line derived neurotrophic factor" (GDNF), and which have been isolated and put into conveniently-handled forms such as plasmids or adenoviral vectors, are described in articles such as Lindsay et al 1996, Choi-Lundberg et al 1997, and Baumgartner et al 1997, and in various other articles cited therein.

Gene sequences which encode "neurotrophin-3" (NT-3) and which have been

isolated and put into conveniently-handled forms such as plasmids or adenoviral vectors, are described in articles such as Snider 1994 and Bothwell 1995, and in various other articles cited therein.

GDNF and NT-3 are regarded as preferred candidates for initial testing for use in treating motor neurons as disclosed herein, since they appear to have relatively strong activity and effects (compared to other known neurotrophic factors) when administered to motor neurons in particular, as indicated by tests done in the prior art.

Numerous gene promoters that drive gene expression in motor neurons are known and available, and can be used in tests such as disclosed herein. One class of promoters that may deserve particular attention for use to transfect spinal motor neurons include the promoters that drive expression of the so-called alpha-1 subunits of glycine receptors, in spinal motor neurons. Since glycine receptors are not present at high concentrations in nocioceptive or other sensory neurons, it is anticipated that use of one or more types of promoters derived from genes which express one or more subunits of such glycine receptors (or other genes that are expressed more actively in spinal motor neurons than in sensory neurons) may help increase selective expression of vector-borne neurotrophic genes in the desired target neurons, while minimizing potential adverse side effects that might be caused by unwanted expression in untargeted cells.

Another class of promoter that might be useful in some situations involving spinal motor neuron transfection drives the expression of a protein known as the polio virus receptor; this receptor protein is not present at substantial levels in sensory neurons, so the promoter is presumed to be silent in sensory neurons. However, it should be noted that many types of lab animals (including mice and rats) do not have polio virus receptor genes or promoters; therefore, it likely would be somewhat more difficult to carry out various types of research in animal models, if polio virus promoters are used.

Alternately, various types of viral and other promoters which are known to be unusually strong promoters in mammalian cells can be used if desired, for purposes such as inducing the highest possible levels of expression of NGF (or other CNS-active polypeptides) in transfected cells. Examples of such strong promoters include the early gene promoter from cytomegalovirus, and the late gene promoter from simian virus-40. Inducible gene promoters can also be used if desired, so long as the inducing factor which activates the selected promoter can be administered in a way which ensures that it will be transported into transfected neurons in adequate quantities.

Accordingly, adenoviral vectors carrying neurotrophic genes that function in motor neurons, such as GDNF or NT-3, can be assembled, using various components and methods as disclosed in the articles cited above.

If desired, an "epitopic tag" sequence can be incorporated into the coding region of the vector gene, to facilitate detection and monitoring of the polypeptide encoded by the vector gene in various tissues of test animals. This approach is discussed in more detail in Example 1, and in articles such as Moller et al 1998. If such an approach is used, it is important to confirm that the polypeptide expressed by the vector construct is able to undergo all steps that are necessary for proper delivery to the neurons that are being targeted for treatment by that polypeptide, using methods described in articles such as Altar & Bakhit 1991, Ferguson et al 1991, DiStephano et al 1992, and von Bartheld 2000.

Adenovirus vectors carrying the desired gene construct can be administered to spinal motor neurons via intramuscular injection, into a lower limb, of adenovirus vector suspended in a volume of solution compatible with adenovirus and tissue vigor (such as physiological saline solution). Methods for propagating, purifying, concentrating, and titering solutions containing adenoviral vectors can be found in publications such as the chapter by Engelhardt (pp. 169-184) in *Methods in Molecular Medicine: Gene Therapy Protocols* (P. Robbins, ed., 1997), and Haase et al 1998 provides information on dosages and administration techniques for efficient administration of adenovirus vectors via intramuscular injection, for transfer of genes into spinal motor neurons in laboratory rats. If desired, electromyographic injection procedures can be used to help ensure that the fluid is injected into the exact desired location.

EXAMPLE 15: MONITORING VECTOR GENES AND POLYPEPTIDES IN TRANSFECTED MOTOR NEURONS, UPPER MOTOR NEURONS, AND BRAIN TISSUE

Tests that use laboratory animals should be designed in a way that will simplify the tasks involved in (i) measuring and monitoring the movement, locations, and concentrations of the genetic vectors and the vector-encoded polypeptides in various cells and spinal cord regions that are likely to be contacted and affected by the polypeptides; and (ii) obtaining reliable and useful statistical data which accurately reflect such results. The necessary tasks can be simplified and rendered more reliable by administering the gene vector to the muscles on one side of a test animal (such as injecting the vector solution into a hind leg),

and treating the other side of the same animal as a control, by using a control vector which carries, for example, an innocuous and/or non-functional gene, a nonsense DNA sequence which does not encode any polypeptide, or a marker gene which encodes a polypeptide that can be easily detected if expressed in mammalian cells, but which has no significant physiological effect. Upon subsequent histological processing, the left and right regions of the spinal cord (and the brain, if desired) can be compared against each other, to assess the movement, concentrations, and effects of the vector DNA and/or the polypeptide(s) encoded by the vector gene(s).

After allowing sufficient time for gene expression (24 to 72 hours), the effectiveness of gene vector delivery can be assessed by removing the lumbar spinal cord from some experimental animals and processing the tissue to monitor for expression of the neurotrophic factor gene within the spinal motor neurons. These types of analyses can use methods such as: (i) hybridization of cellular mRNA with DNA probes that are complementary to the gene vector mRNA, but not to endogenous mRNA sequences, using procedures as described in articles such as Xian and Zhou 2000; (ii) techniques which use "polymerase chain reaction" (PCR) reagents and methods to detect DNA or mRNA sequences from the viral vector, as described in articles such as Chie et al 2000; and, (iii) immunostaining, ELISA, or similar methods which use antibodies that selectively bind to the vector polypeptide but not to the endogenous polypeptide in the test species. Many such antibody preparations are commercially available; alternately, if desired (such as to detect polypeptides having a specific "epitopic tag"), they can be prepared using methods disclosed in articles such as Conner 2000, Rush et al 2000, and Zhang et al 2000.

The goal of the procedures outlined in Examples 14 and 15 is to use transfected spinal motor neurons which straddle the blood-brain barrier to deliver therapeutic polypeptides to the upper motor neurons that lie protected wholly within the BBB. This is achieved by: (i) creating vector-encoded neurotrophic polypeptides, by expressing the vector-borne neurotrophin genes, and then, (ii) transporting and delivering those polypeptides to locations in the spinal cord which are protected from foreign polypeptides by the BBB, but which are accessible to axonal processes of upper motor neurons.

To facilitate the process of identifying the exact regions within the spinal cord where such polypeptides are most likely to be delivered by transfected spinal motor neurons, "transneuronal labelling" studies can be carried out, using appropriate tracer procedures and reagents. Such studies which made use of the pseudorabies virus are described in articles

such as Card et al 1990.

Transneuronal labelling can also be used to obtain evidence of delivery of polypeptides such as NT-3 or GNDF to the upper motor neurons resulting in upper motor neuron sprouting and establishment of synaptic connections with the transfected BBB straddling lower motor neuron. The number of transneuronally labelled upper motor neurons in the brain and brainstem will be greater by virtue of an increased strength and number of synaptic contacts between upper and lower motor neurons.

Where a control and test vector are administered to opposite sides of the experimental animal, appropriate differences between the left and right sides of the spinal cord will be observed. Severing the descending axonal tracts in some animals can be used to confirm that the NT-3 or GDNF detected in the brain in other experimental animals was derived from retrograde transport from transfected lower motor neurons.

EXAMPLE 16: MONITORING PHYSIOLOGICAL AND MUSCULAR EFFECTS CAUSED BY DELIVERING NEUROTROPHIC FACTORS TO UPPER MOTOR NEURONS

The physiological effects of administering neurotrophic factors such as NT-3 or GDNF into the spinal cord or brain, using genetic vectors as disclosed herein, include effects on the neuroanatomy of the spinal cord and brain, and effects on the physiology and behavior of the animal. In particular, improvements and benefits in muscle strength, muscle control, and muscle tone that can be provided by using the genetic vectors disclosed herein can be evaluated using various methods.

These methods require a basic understanding of what has previously been seen in various prior tests using animal models. It must also be recognized that most such tests are necessarily carried out by first inflicting some sort of neuronal damage or injury upon the spinal cord or motor neuron system of an animal, then waiting for some period of time for the injury to be more fully manifested, then administering some sort of test treatment (such as direct infusion of a neurotrophic factor into the spinal fluid of the animal, using a hypodermic needle), and finally, by subsequent testing of the animal to determine whether the test treatment was able to reduce the extent of damage that was inflicted by that same type of injury in control animals or limbs. As summarized in Example 4, comparative tests usually involve either or both of the following: (i) different control animals, or (ii) treatment of the two different sides of the same animal in different manners.

It should be recognized that many such tests use "axotomy", which refers to surgically severing the axon. As noted in Example 4, the axon is crucial to a neuron's functioning, and over a span of time measured in days, if a neuron's axon has been severed in a location that is relatively close to the neuron's cell body, the neuron likely will begin to atrophy and will eventually die, even though the entire remainder of the neuron is undamaged. The reasons for this are complex, and are generally believed to involve cellular factors (and especially neurotrophic factors) involved in nervous system development. According to the so-called "neurotrophic hypothesis", a developing brain in a fetus initially generates an oversupply of neurons, then a pruning process begins. During the pruning stage, neurons that do not actively continue to receive incoming nerve signals (and/or are not contacted by one or more types of neurotrophic factors) die off, in a form of programmed cell death called "apoptosis."

Many animal studies have demonstrated that application of various neurotrophic factors (usually by injection into spinal fluid or spinal tissue) can prevent the type of atrophy, degeneration, and death that can be induced in upper motor neurons by axotomy injury. Examples of such animal tests, and the results that arise when neurotrophic factors are applied to such neurons, are reported in articles such as Novikova et al 2000, Giehl and Tetzlaff 1996, and Giehl et al 1997.

However, where the axotomizing injury is distant from lower motor neurons that have been genetically transfected with foreign genes (as will occur if and when the genetic vectors disclosed herein are used in the manner disclosed in Example 14), it will not be easy to demonstrate that the vectors and genes of this invention had an effect on axotomy-induced atrophy or degeneration, because the axotomy injury will interfere with the normal retrograde axonal flow of neurotrophic factor within the transfected peripheral motor neuron. If the transfected lower motor neurons lie within a few millimeters of the severed end of the upper motor neuron, the upper motor neuron is more likely to have access to the neurotrophic polypeptide that is expressed and secreted by transfected motor neurons. Accordingly, appropriate experimental designs which use axotomy injuries preferably should use an axotomic locations that are within reasonably close distances to the closest tips of the motor neurons that can reasonably be transfected by using muscle injections.

Administration of neurotrophic factors has also been shown, in published reports, to stimulate sprouting and regeneration of injured axons (e.g., Schnell et al 1994). Accordingly, anterograde tract tracing studies, involving injection of tracer compounds such

as biotinylated dextran into the motor cortex (as described in Ferguson et al 2001) may be used to study and demonstrate various effects (such as stimulation of sprouting and regeneration) when genetic vectors such as disclosed herein are used to deliver factors such as NT-3 or GDNF via lower motor neurons that lie within about 1 to 5 mm of the severed end of an injured upper motor neuron.

Use of the invention to deliver NT-3 or GDNF via lower motor neurons is also expected to establish a chemoattractant gradient, where the highest concentrations of the attractant compound are likely to be centered near the transfected lower motor neurons (unless such molecules are rapidly cleared or dispersed, such as by active uptake into other cells). Regenerating injured motor neurons typically will respond to this type of chemoattractant gradient, by growing in the direction of increasing concentrations of the chemoattractant. By making use of anterograde tract tracing methods, such as described in Ferguson et al 2001, the influence of such chemoattractant gradient on the directional growth of the regenerating injured upper motor neurons may be evaluated and demonstrated.

Establishment of a chemoattractant gradient emerging from transfected lower motor neurons is also likely to accelerate the rate at which regenerating injured upper motor neurons will form new synaptic connections with the neurons that are releasing a chemoattractant compound, such as GDNF or NT-3. This type of acceleration in the rate of synaptic connection can be demonstrated by undertaking time-course studies in animal models, and monitoring the time course of appearance of a transneuronal tracer compound in the upper motor neuron, if the tracer was injected into regions outside the BBB. Suitable transneuronal tracer methods are described in articles such as Ugolini 1995, Travers et al 1995, and Card et al 1990.

Establishment of functional synaptic connections between upper and lower motor neurons also will alter the electrical behavior of the lower motor neurons. The primary effect of upper motor neuron activity tends to be inhibitory: therefore, when this form of inhibition is lost, lower motor neurons tend to enter a hyperactive status. Accordingly, restored or regenerated connections with upper motor neurons is expected to reestablish more normal levels of activity in the lower motor neurons, which will manifest as more normal sensory reflexes.

Electrophysiological methods can be used to monitor the time course of such changes, and can provide an indicator of whether functional synaptic reconnections are

being established between upper and lower motor neurons.

Establishment of functional reconnection between upper and lower motor neurons will also result in observable changes in motor function, including muscle strength, muscle tone, and coordination. The time course of change in muscle strength, and the ability of an animal to perform fine motor or coordinated motor tasks (such as by overcoming challenges in order to obtain food), can be tested and monitored by methods described in the literature and known to those skilled in the art.

EXAMPLE 17: USE OF LENTIVIRUS-DERIVED VECTORS FOR TRANSFECTING SPINAL MOTOR NEURONS THAT WILL TRANSPORT NEUROTROPHIC POLYPEPTIDES TO UPPER MOTOR NEURONS

The methods described in articles such as Hottinger et al 2000 can be used to construct a lentivirus vector capable of carrying a gene construct which has (i) a GDNF, NT-3, or other neurotrophic polypeptide coding sequence, and (ii) a gene promoter suited for gene expression in transfected spinal motor neurons, as described in Example 14. This type of lentivirus-derived gene vector may be administered to the accessible projections of spinal motor neurons via intramuscular injection, using procedures such as described in Example 14, above.

After transfection of the spinal motor neurons, expression of the encoded polypeptide in the transfected neurons, and delivery of the polypeptide into spinal cord or brain tissue protected by the BBB, can be measured and monitored by methods such as described in Example 15, above. Muscular and other physiological effects of the neurotrophic polypeptide, and the ability of the neurotrophic polypeptide to prolong neuronal survival following axotomy or similar challenge, can be measured and monitored by methods such as described in Example 16, above.

EXAMPLE 18: PREPARATION AND USE OF LIPOSOME VECTORS FOR TRANSFECTING SPINAL MOTOR NEURONS THAT WILL TRANSPORT NEUROTROPHIC POLYPEPTIDES TO UPPER MOTOR NEURONS

As noted in Example 6, methods for preparing DNA plasmid-lipid complexes for transfecting mammalian cells are described in publications such as the chapter by Nabel (pp. 127-133) in *Methods in Molecular Medicine: Gene Therapy Protocols* (P. Robbins, ed., 1997), and can be adapted for cell types or specialized uses by routine testing of various

preparative mixtures.

Liposomal vectors carrying neurotrophic factor genes may be administered to the accessible projections of spinal motor neurons via intramuscular injection, using procedures such as described in Example 14, above. After transfection of the spinal motor neurons, expression of the encoded polypeptide in the transfected neurons, and delivery of the polypeptide into spinal cord or brain tissue protected by the BBB, can be measured and monitored by methods such as described in Example 15, above. Muscular and other physiological effects of the neurotrophic polypeptide, and the ability of the neurotrophic polypeptide to prolong neuronal survival following axotomy or similar challenge, can be measured and monitored by methods such as described in Example 16, above.

EXAMPLE 19: CONSTRUCTION AND USE DNA VECTORS THAT TARGET ENDOCYTOTIC RECEPTORS ON SPINAL MOTOR NEURONS THAT WILL TRANSPORT NEUROTROPHIC POLYPEPTIDES TO UPPER MOTOR NEURONS

Reagents and procedures for preparing genetic vectors using ligands which bind to endocytotic receptors on neurons are discussed in Example 7, above. Those procedures include methods for using monoclonal antibodies, or repeated selection cycles involving phage display libraries, to identify and create high-affinity ligands that will actively bind to endocytotic receptors on spinal motor neurons.

As introduced in Example 7, ligands to the p75 receptor can be used to target gene delivery to the spinal motor neurons. While spinal motor neurons normally express only low levels of p75, these spinal motor neurons upregulate their expression of p75 in response to injury or deprivation of neurotrophic factors, and in various diseases such as amyotrophic lateral sclerosis. Therefore, a p75 targeting gene vector enables enhanced or targeted delivery of genes encoding therapeutic proteins to spinal motor neurons in need of therapeutic support.

The efficiency of gene vectors which target spinal motor neurons can be enhanced by including, in the vector construct itself or in an injectable solution which contains the gene construct, a compound that can concentrate the gene vector at the "motor endplate" in an injected muscle. For this purpose, botulinum toxin may be useful; alternately, a monoclonal antibody may be generated, using a 17-amino acid peptide sequence from the α_1 subunit of the acetylcholine receptor as the antigen (see Yoshikawa et al 1997). In a disease called myasthenia gravis, a patient develops antibodies against this epitope of the

acetylcholine receptor, and these antibodies are localized on the muscle endplate in the synaptic cleft.

Accordingly, "receptor-targeting" gene vectors which use such receptor-binding ligands and possibly other enhancements can be used to carry gene constructs that have a GDNF, NT-3, or other neurotrophic polypeptide coding sequence, and a gene promoter suited for gene expression in transfected spinal motor neurons, as described in Example 14. Administration to spinal motor neurons via intramuscular injection of ligand-DNA complexes can use procedures such as described in Example 14, above. After transfection of the spinal motor neurons, expression of the encoded polypeptide in the transfected neurons, and delivery of the polypeptide into spinal cord or brain tissue protected by the BBB, can be measured and monitored by methods such as described in Example 15, above. Muscular and other physiological effects of the neurotrophic polypeptide, and the ability of the neurotrophic polypeptide to prolong neuronal survival following axotomy or similar challenge, can be measured and monitored by methods such as described in Example 16, above.

EXAMPLE 20: TRANSFECTION OF SPINAL MOTOR NEURONS USING TRANS-NEURONAL VECTORS TO DELIVER NEUROTROPHIC FACTOR GENES TO CNS NEURONS IN CONTACT WITH THE SPINAL MOTOR NEURONS

Genetic vectors that may have "transneuronal" transport capability are described in Example 8, above. Such vectors designed for use in transfecting spinal motor neurons can be assembled using methods such as described in Example 8, and can carry gene constructs such as described in Example 14, above. Administration to spinal motor neurons via intramuscular injection can use procedures such as described in Example 14, and post-transfection monitoring can use methods such as described in Examples 15 and 16.

EXAMPLE 21: DELIVERY OF NEUROTROPHIC FACTORS INTO THE BRAINSTEM, BY INJECTION OF VECTORS INTO THE TONGUE TO TRANSFECT LOWER MOTOR NEURONS OF THE HYPOGLOSSAL NUCLEUS

Genetic vectors derived from adenoviruses, herpes viruses, or lentiviruses, or using cationic liposomes, ligands that bind to endocytotic receptors, and/or trans-neuronal polypeptides, can be created as described in the examples above. These types of vectors can carry neurotrophic gene constructs (or, if desired, gene constructs that express recombinant antibodies or similar polypeptides that inhibit one or more neurite inhibitory molecules such

as IN1 or No-Go) which will be expressed in transfected motor neurons, as described in the foregoing examples.

As illustrated in FIG. 6, such vectors can be used to transfect a certain class of lower motor neurons which have projections that are accessible outside the BBB, in the tongue. These neurons, known as "motor neurons of the hypoglossal nucleus," are synaptically connected to other neurons in certain regions in the brainstem. Therefore, the motor neuron terminals inside the tongue offer a relatively direct passageway for delivering neurotrophic polypeptides into neurons of the brainstem that synapse with or have projections that lie near these transfected lower motor neurons.

Administration to these motor neurons can be achieved via injection into the tongue, using general procedures such as described in Example 14 but adapted for injection into the muscles of the tongue. Post-transfection monitoring in test animals can use general methods such as described in Examples 15 and 16, appropriately adapted to monitor for delivery of exogenous peptides (such as epitope-tagged NGF, NT-3, or GDNF) to neurons in the brain stem, as well as other methods known to those skilled in the art. As one example, for use in animals, various transneuronal tracer methods, such as described by Ugolini 1995, Travers et al 1995, or Card et al 1990, can be used to label neurons in brainstem which make contact with transfected lower motor neurons in the hypoglossal nucleus. By counting the number of second-order neurons which contact the hypoglossal nucleus in animals treated with the invention, and comparing such data to numbers seen in control animals, evidence can be obtained of the delivery of neurotrophic factor to second-order neurons in the brainstem.

As another example of a monitoring method which may be useful in human patients, hyperexcitable reflex blinks are a symptom of Parkinson's disease, which correlates clinically with the severity of the disease. Tonically active serotonergic "raphe neurons" normally inhibit the spinal trigeminal neurons involved in reflex blink circuits, and deterioration of these serotonergic raphe neurons leads to the hyperexcitable blink reflex (e.g., Basso and Evinger 1996). Accordingly, evidence of the effectiveness of clinical use of the invention to reduce the rate of degeneration of serotonergic neurons of the brainstem can be obtained by monitoring the hyperexcitable blink reflex in patients suffering Parkinson's disease.

Another of method for monitoring preservation of brainstem neurons in human patients with Parkinson's disease involves taking measurements of a patient's swallowing

reflex. The swallowing reflex involves a coordinated movement of tongue and other oral and respiratory muscles and, in Parkinson's disease, the time between stimulus and reflex swallowing is abnormally prolonged. A swallowing reflex test involves delivery of a small volume of water into the throat, via intranasal catheter, as the swallowing reflex stimulus. The initiation of swallowing reflex can be accurately measured using surface electrode electromyographic recording of the muscles in the throat and used to calculate the time between stimulus and reflex swallowing (Iwasaki et al 2000). Accordingly, evidence of the effectiveness of clinical use of the invention to reduce the rate of degeneration of neurons of the brainstem can be obtained by monitoring the swallowing reflex in patients suffering Parkinson's disease.

EXAMPLE 22: IN VIVO SCREENING: PHAGE TYPES AND LIBRARIES

M13KO7 helper phages can be purchased from various commercial suppliers, such as New England Biolabs (www.neb.com) and Amersham Biosciences (www4.amershambiosciences.com). This strain of helper phage contains fully functional genes that encode both the pIII and pVIII coat proteins. It also contains an origin of replication (from plasmid p15a) which is tightly controlled in a manner that results in low copy numbers in bacterial cells. It also contains a mutated (Met-40-Ile) copy of the phage M13 pII gene, which is essential for phage replication; this mutation causes it to be secreted by *E. coli* cells, as phage particles, in low copy numbers. It also carries a kanamycin resistance gene, inserted at the Ava I site within the M13 origin of replication. This kanamycin gene functions as a selectable marker in *E. coli* host cells that are not resistant to kanamycin. Additional information on using and culturing these helper phages is available from commercial suppliers, and in various published articles describing their use.

The scFv phage library was supplied by Cambridge Antibody Technology (Cambridge, England; www.cambridgeantibody.com). It is described in various patents (such as US 6,172,197) and published articles. It was created by inserting gene sequences obtained from B-lymphocyte cells (which create antibodies) into the gene sequences that encode the pIII coat protein (which is located, in relatively small numbers, at one end of filamentous M13 phage particles). Each foreign gene sequence in the scFv library contains both the "heavy variable" (V_H) and "light variable" (V_L) domains of a single antibody, in a single gene sequence that will express the V_H and V_L domains in a "single chain" (sc) polypeptide, having an average molecular weight of about 35 kilodaltons. The scFv library

has an estimated 13 x 10^9 (i.e., 13 billion) different recombinants. To ensure maximal diversity, it contains "variable fragment" (Fv) antibody domains obtained from numerous people of different ancestries. The library is estimated to encode a range and diversity of different Fv antibody domains that could be generated by the immune systems of ten different people from a varied assortment of racial and ethnic groups.

It should also be noted that the scFv phages are phagemids. They have a bacterial origin of replication, which causes them to reproduce in high copy numbers, as double-stranded plasmids, in *E. coli* cells. They also contain a phage origin of replication, which can trigger the synthesis of single-stranded DNA for assembly into phage particles; however, that ssDNA synthesis requires a phage ssDNA transcribing protein to be present, and scFv phages do not encode that protein. That ssDNA transcribing protein must be supplied by helper phages, such as the M13KO7 helper phages mentioned above.

Therefore, when M13KO7 helper phages are used to coinfect *E. coli* cells that have already been infected by scFv phagemids, the addition of the ssDNA transcribing protein (from the helper phages) to cells that already contain large numbers of dsDNA plasmids (from the scFv phagemids) will trigger the formation of large numbers of ssDNA strands, from the scFV phagemid plasmids. These newly formed ssDNA strands will then be packaged inside coat proteins (mainly pVIII coat proteins from a particular scFV clone which infected that host cell). The newly packaged ssDNA and its coat proteins will secreted by the host cell, as filamentous phage particles. Most of these secreted phage particles will contain scFv phagemid DNA, rather than M13KO7 helper phage DNA, since the helper phage DNA sequences will be present in the host cells only in low copy numbers (due to the low-copy-number plasmid p15a origin of replication in the helper phage DNA).

The pVIII coat proteins in the phage particles that are secreted by a some particular *E. coli* host cell will contain clonal copies of some particular antibody "variable fragment" polypeptide sequence, which was encoded by a DNA sequence that was obtained from a human B-lymphocyte. This human antibody DNA sequence was inserted into the scFv phagemid DNA at a controlled and targeted site, near the middle of the phagemid gene that encodes the pVIII coat protein.

The Ph.D-C7C phage display library was obtained from New England BioLabs (www.neb.com, catalog number 8120). This library contains an estimated 2 x 10^9 different recombinants, with foreign DNA inserts encoding random sequences of seven amino acid, inserted near the DNA sequence that encodes the N-terminus of the pIII coat protein of

M13 phages. This library provided an essentially random repertoire of peptide sequences that could be tested, to determine whether certain phages would be internalized and transported by neurons in the sciatic nerve bundle.

EXAMPLE 22: CELL TYPES, TRAITS, AND METHODS

Except as otherwise noted, all phage amplification and titering used the TG1 strain of *E. coli*, from Cambridge Antibody Technology. This strain, which was specifically designed and developed for working with M13 phages, is also sold by companies such as Stratagene (La Jolla, California; www.stratagene.com). Additional information describing culturing and transformation methods for this strain can be downloaded at no cost from the websites of commercial suppliers.

Among other features, the TG1 strain has a "lacIq" repressor gene which, together with catabolite repression by glucose, negatively regulates a "lac" promoter that has been placed in control of expression of the M13 gene that encodes the pIII phage coat protein. Most types of M13 phages that are used with TG1 cells contain an "amber" stop codon, inserted at the start of the pIII gene. As described below, this allows expression of pIII polypeptides (including chimeric pIII polypeptides that contain foreign amino acid sequences) in soluble form, in a non-suppressing *E. coli* strain such as HB2151, without having to reclone the gene.

The "amber" stop codon can be effectively inactivated by transferring TG1 cells into culture medium that contains no glucose, and that instead contains lactose (a particular type of sugar molecule) as the sole source of carbon for the bacteria. A compound called iso-propyl-thio-galactopyranoside (IPTG) is also added; this potently induces expression of the "lac" operon, which enables the host cells to metabolize lactose molecules as a nutrient. In addition to enabling transformed cells to grow in media with lactose as a sole carbon source, it also enables transformed cells to convert a chemical called X-Gal into a bright blue color, so that transformed colonies can be easily identified and isolated, on agar plates.

In a typical procedure used to "amplify" (reproduce) a particular assortment of phages (such as after an *in vitro* panning or in vivo selection procedure as described herein), a colony of TG1 cells that had been grown on an agar plate was used to inoculate liquid culture media which contained 16 g tryptone, 10 g yeast extract, and 5 g NaCl per liter (this type of liquid culture media, containing tryptone and yeast extract, is referred to as 2TY media). The cells were replicated in a shaking incubator to an optical density

(OD_{600}, measured at a light wavelength of 600 nanometers) of about 0.5 to 0.8 units (all incubations were done at 37°C, unless otherwise indicated). A phage preparation was added to the *E. coli* culture, and the mixture was incubated. Initial incubation was carried out in stationary conditions, for 30 minutes, to facilitate binding of the phages to the bacterial cells. This was followed by 30 minutes in a shaking incubator running at 200 rpm, to ensure maximal exposure of the cells to fresh nutrients.

These cells were then centrifuged at 3500 rpm for 10 minutes, and the supernatant containing old broth and metabolites was discarded. The cell pellet was resuspended in 500 microliters (μL) of fresh 2TY culture broth, and the mixture was spread across the surfaces of four fairly large (24.3 cm x 24.3 cm) square plates containing 2TY agar media with ampicillin and glucose. The plates were incubated overnight at 30°C. Because ampicillin was present, only *E. coli* cells that contained scFv phagemids or PhD-C7C phages gave rise to colonies on the plates.

The following day, to complete the preparation of standardized phage solutions that could be frozen until needed, colonies were scraped from each agar plate into 10 mL of 2TY broth, in a 50 mL tube. A half-volume of sterile 100% glycerol was added, and the solution was mixed by placing the tube in an end-over-end rotator for 10 minutes at room temperature. 1 mL aliquots were frozen at -70°C for storage.

When a batch of phages was needed for a test, a 1 mL aliquot of the glycerol-containing stock was thawed, and 100 μL of the thawed stock was added to 25 mL of 2TY broth containing 2% (w/v) filter-sterilized glucose and 100 mg/mL ampicillin. The cells were grown at 37°C in a shaking incubator until they reached an OD_{600} density of about 0.5 to 0.8. M13KO7 helper phages were then added, to form a final concentration of 5×10^9 "colony forming units" (cfu) per mL. The mixture was incubated for 30 minutes while stationary, then for 30 minutes in a shaker tray at 200 rpm. The cells were then centrifuged at 3500 rpm for 10 minutes, and the cell pellet was resuspended in 25 mL prewarmed 2TY (without glucose) containing kanamycin (50 μg/mL) and ampicillin (100 μg/mL). These were incubated overnight at 25°C, with rapid shaking, to produce phage particles.

The phage particles were purified from the supernatant by precipitation with 20% polyethylene glycol (PEG) and 2.5 M NaCl. These particles were then resuspended in a final volume of 1.5 mL of sterile phosphate buffered saline (PBS) at about 4°C.

To "titer" a solution that contains phage particles (i.e., to obtain an estimate of how many infective phage particles were present in each mL of solution), TG1 cells were grown

in 2TY media, in a shaking incubator at 300 rpm for about 4 hrs, until an OD_{600} density of about 0.5 to 0.8 was reached. A sample of phage supernatant was serially diluted at 10-fold dilutions, in 2TY media, by adding 50 μL of each dilution in the series to a 450 μL suspension of TG1 cells in an Eppendorf tube. The tube was incubated stationary for 30 minutes, followed by shaking at 300 rpm for 30 minutes. 100 μL of each dilution of the infected TG1 cells were streaked onto prewarmed 2TY agar plates (2TY media containing 100 μg/mL ampicillin, 2% w/v filter-sterilized glucose, and 1.5% w/v agar). The plates were incubated overnight, and the following day, the number of colonies were counted. TG1 cells could grow on ampicillin-containing media only if they carried ampicillin resistance genes from a phage.

EXAMPLE 24: CROSS-LINKING OF P75 RECEPTOR-BINDING ANTIBODIES (MC192) TO M13KO7 HELPER PHAGES

A monoclonal antibody preparation known as MC192 (and by similar terms, such as clone 192; originally described in Chandler et al 1984) is commercially available from various suppliers, such as Cell Sciences (www.cellsciences.com) and Chemicon (www.chemicon.com). These monoclonal antibodies bind to "low affinity" (p75) nerve growth factor receptors on rat neurons. Monoclonal antibodies that bind to human p75 receptors are also available, from companies such as United States Biological (www.usbio.net).

Unlike various other monoclonal antibodies that also bind to p75 receptors in rats, the MC192 antibody can trigger endocytosis of the antibody-receptor complex, leading to neuronal uptake of the MC192 antibody. This has been shown by studies using radiolabelled antibodies (Johnson et al 1987, Yan et al 1988).

To evaluate the ability of the MC192 antibody to drive endocytosis of phages into rat neurons, a preparation was made, containing MC192 antibodies that were chemically crosslinked to M13KO7 helper phages, using a multi-step process. First, sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (abbreviated as sulfo-SMCC; purchased from the Pierce company, Australia) was reacted (through the active sulfo-NHS ester end) with primary amine groups on the MC192 antibody. This resulting in the formation of an amide bond between the antibody and each cross-linker group, with sulfo-NHS being released as a byproduct. To remove unreacted sulfo-SMCC, the activated antibody was purified using Microcon YM-100 centrifugal filter (100 kilodalton cut-off; catalog number

42412, Millipore Corporation, USA).

In the next phase, M13KO7 phages were incubated with 2-iminothiolane (2-IT; also called Traut's reagent) to generate free sulfhydryl groups; these groups are positioned at the ends of short chains that are bonded to lysine residues, in the pVIII coat protein of the phages. The phages were filtered, using YM-100 kDa filters, to remove excess reagent.

The antibody preparation was then mixed with the phage preparation, at a 1:10 ratio, to form thioester crosslinking bonds, from the maleimide groups on the activated antibodies and the sulfhydryl groups on the activated phages. The reaction product was filtered to remove excess antibodies, and iodoacetamide (Sigma Chemical) was added to block any remaining free reactive sulfhydryl groups. The reaction product was precipitated twice in PEG/NaCl (20% w/v polyethylene glycol, average molecular weight 8000, in water with 2.5 molar NaCl) to remove free antibodies.

The resulting phage mixture contained various numbers of MC192 antibodies, located randomly along the length of the phage. Because of the 10:1 ratio of antibodies to phages in the reaction mixture, it was presumed and estimated that on average, from about 2 to about 20 antibodies were bonded to most phage particles.

EXAMPLE 25: SURGICAL TREATMENT AND PHAGE EMPLACEMENT IN RATS

Female Sprague-Dawley rats were used, and surgeries were performed under halothane anaesthetic (2% in oxygen, administered by nose cone connected by tubing to anesthetic machine). Alternatively, longer-acting injectable anesthetics such as sodium pentobarbitone may be used if desired.

Certain comments are offered below, about preferred procedures for doing this type of surgery on rats, since the use of correct procedures will substantially increase the likelihood of success. Many people who are quite familiar with cells and phages may not be familiar with small animal surgery, as is necessary to carry out the *in vivo* procedures of this invention.

It should also be noted that whenever someone is doing this type of surgery for the first time, a binocular microscope that can provide up to 20x magnification is almost always used, during training, to help focus the vision and attention on important sites and aspects of the procedure. After conducting a procedure a few times, a technician can choose whether or not to use a microscope during subsequent procedures (or during a delicate or difficult part of a procedure, such as suturing the two ends of a sciatic nerve together, after

placing the phage-containing gel foam between the nerve ends). If mice are used, their smaller size might dictate use of a binocular microscope for all procedures, until a technician develops a fairly high level of experience and familiarity with the procedures.

When the rats were 6 weeks of age, an initial surgery was performed, to "upregulate" (increase) expression of the p75 cell receptors. If the sciatic nerve is injured in this manner, the motor neurons (which have their cell bodies in the spinal cord, and axonal fibers projecting through the sciatic nerve) are stimulated to express increased numbers of p75 receptors on their cell and axonal surfaces. Tests that were conducted to compare the differences between phage uptake by pre-ligated neurons, versus phage uptake by non-ligated neurons, indicated that the pre-ligation step increased p75 receptor density and phage uptake by roughly 13-fold. These tests included phage uptake tests, as well as staining (using MC192 antibodies) of tissue sections taken from the lumbar regions of spinal cords of rats.

Two other factors should also be noted about p75 receptors, in rats. First, it is present in substantial numbers on the surfaces of motor neurons that originate in the spinal cord, and that send out axons or other neuronal fibers into muscle tissues that are not enclosed within the blood-brain barrier. This includes sciatic nerves; however, the p75 receptor is present in substantial copy numbers, on sciatic nerve surfaces, for only about two weeks after a rat is born, while the rat is growing rapidly. After about one to two weeks, its copy number on sciatic nerves drops off, and by the time rats are about 6 weeks old, it is present on sciatic nerve surfaces only in very low and often undetectable quantities.

The second notable factor is this: since p75 receptors are not abundant on sciatic nerve surfaces in rats that are 3 weeks old or older, even after a controlled injury has been inflicted on a sciatic nerve, the p75 receptor endocytosis system can be saturated, fairly easily. It apparently was saturated, on a number of occasions, during the *in vivo* screening tests described herein. However, rather than invalidating any of the results disclosed herein, this factor should be regarded more as a "ceiling" value, which cannot be exceeded. Accordingly, these saturation limits can be approached and utilized in ways that appear to confirm and validate the mechanisms and effects that are believed by the Applicants to be active in these types of *in vivo* screening tests using p75 receptors.

During the initial surgery, there is no need to use any mechanical restraint. The animal is laid on its side with the hindlimb uppermost, fur shaved and skin swabbed. A 1 to

3 cm midthigh skin incision in parallel with the femur is made, using surgical scissors or scalpel. Using the tip of closed surgical scissors (blades 2 to 4 cm long), the femur is located by palpation, and the point of the scissors is pushed, just caudal to the femur, through the muscle layers to a depth of 1 to 2 cm, depending on the size of the animal. The scissors are then opened, to separate the muscle with minimal bleeding, and to create a 1 to 2 cm window which exposes the sciatic nerve lying beneath the muscle. Retractors or sutures can be used to hold open the muscle and maximize the window of operation, but this may not be required by an experienced technician.

By using this entry procedure, the sciatic nerve can be clearly seen. The sciatic nerve is only loosely attached to the surrounding tissues, by membranes that are easily separated. The nerve itself is protected by a tough nerve sheath, and an estimated 50,000 axons may be contained within this nerve bundle, depending on the location. While the axons of some sympathetic or other nerve may not be myelinated, each motor axon (and most sensory nerve axons) is surrounded by a myelin sheath, contributed by Schwann cells, which make up the bulk of nerve tissue mass outside the blood brain barrier.

A ligature is emplaced by inserting a pair of curved forceps under the sciatic nerve, and used the forceps to gently lift the nerve and free any loosely adhering membranes, if present, from a 1 to 2 cm length. The forceps are opened and used to grasp a length of 6/0 silk suture, which is then pulled under the nerve by withdrawing the forceps. The suture, which is placed at a site slightly above the location where the tibial branch bifurcation divides the sciatic nerve bundle into two smaller bundles, is then tied tightly around nerve to ligate it. It is important to use non-resorbable sutures, such as silk or nylon. Black silk is generally preferred, since it is less elastic (making tight ligations easier to secure), and because a black ligature is more easily located during a subsequent operation.

The instruments are withdrawn, the separated muscles are allowed to rejoin, and the skin incision is closed with 1, 2 or 3 sutures, depending on the length of the incision. The animal is then allowed to recover from anesthesia.

Seven days later, the sciatic nerve was exposed again, and a 2 to 3 mm section of the sciatic nerve which contained the ligature was excised, using a pair of surgical scissors.

Roughly 9 cubic millimeters of a collagen matrix gel foam, containing 10 μl of the MC192-M13KO7 antibody-phage conjugate (with titers ranging from about 3.3×10^6 to about 2.1×10^9 cfu/mL) was inserted between the two transected ends of the nerve bundle.

The free ends of the sciatic nerve were sutured together, flanking the gel foam that

contained the phages, using a 10-0 nylon surgical suture. A small flexible sleeve of silicone rubber was placed around the nerve ends and the gel foam containing the antibody-phage conjugates, to ensure that the antibody-phage conjugates in the gel foam would remain in direct contact with the ends of the nerve fibers.

During the same surgery when the transection and phage emplacement were made, the sciatic nerve bundle was ligated, using a 6-0 silk suture, at a location about 2 cm above the transection site, near the rat's hip. This ligature is referred to herein as the hip ligature, to distinguish it from the initial ligature that was used to increase p75 receptor expression.

The hip ligature created a constriction point that prevented antibody-phage conjugates that had been taken into sciatic neurons from being retrogradely transported all the way to the spinal cords of the neurons. Therefore, antibody-phage conjugates accumulated, inside the nerve fibers, at a location that was just below (distal to) the hip ligature.

After a delay of 18 hours, to allow enough time for endocytotic uptake and retrograde transport, the rat was sacrificed by chloroform inhalation, and a nerve segment distal to the hip ligature was harvested, as disclosed in the next example.

EXAMPLE 26: NERVE HARVESTING AND INTERNALISED PHAGE COLLECTION

As mentioned in the prior example, a rat was sacrificed 18 hours after: (i) emplacement of the collagen gel containing the phage particles, and (ii) emplacement of the hip ligature.

A nerve segment which included the hip ligature and a segment of nerve fibers just distal to that ligature was harvested. This was done by emplacing and tightening an additional ligature around the sciatic nerve, about 0.5 cm below (distal to) the hip ligature, to prevent any loss of the phage particles from either of the cut ends of the nerve bundle. A segment of the sciatic nerve bundle, which contained both of the two ligature loops still tied tightly around both ends of the segment, was then cut out and removed.

The excised nerve bundle was scrubbed 3 times with sterile PBS, using forceps with sterilized tissue paper, until the outer membrane was removed. The neurons were then transferred onto a dry glass plate, and the ligatures were removed.

450 μL of a lysis buffer (which digested cell membranes but not bacteriophage particles) was then applied, containing 1% Triton X-100, 10 mM Tris, and 2 mM EDTA at

pH 8, and also including 1/100 (by volume) of a protease inhibitor mixture (containing 4-(2-amino-ethyl)-benzenesulfonyl fluoride, pepstatin A, E64, bestatin, leupeptin, and aprotinin in dimethylsulfoxide, purchased from Sigma Chemical, Australia). While in the lysis buffer, the nerve fibers were cut into small pieces, using a scalpel, and the resulting suspension was transferred to an Eppendorf tube and incubated at room temperature on vortex for 1 hour. The tube was then centrifuged at 10,000 rpm at 4°C for 10 minutes, to pellet the sciatic nerve debris.

The supernatant (which contained phage particles) was collected and stored on ice, while the debris pellet was incubated with 300 μL more lysis buffer and vortexed for 1 more hour at room temperature. The lysed debris was then incubated at room temperature for 1 hour, and the sample was transferred into another Eppendorf tube and centrifuged at 10,000 rpm at 4°C for 10 minutes, to pellet any remaining debris. The supernatant was collected and added to the previous supernatant, and a 20% volume of $CaCl_2$ was added, to inactivate the EDTA in the lysis buffer.

Some of the resulting aliquots of the mixed supernatants were titered, as described in Example 25, to determine phage particle concentrations. Other aliquots had a 50% volume of glycerol added, and the mixture was frozen and stored at -20°C for subsequent use or analysis. During all but the final rounds of *in vivo* selection, still other aliquots were used to infect *E. coli* cells, and the amplified phage preparations that resulted were used as reagents in subsequent cycles of *in vivo* selection, using the same procedures described above.

EXAMPLE 27: HISTOLOGIC PHOTOGRAPHS OF NERVE SEGMENTS

A fluorescent staining technique was used to generate photomicrographs that visually confirmed the accumulation of internalised and transported MC192-M13KO7 antibody-phage conjugates in the sciatic nerve bundle, just below the hip ligature.

To create these photographic confirmations, the animal was euthanised with an overdose of anesthetic (sodium pentobarbitone, 80 mg/kg, injected into the abdomen IP). It was then perfusion-fixed through the heart, using 400 mL of ice cold 0.1 molar sodium phosphate buffer containing 2% paraformaldehyde and 0.2% parabenzoquinone over 30 minutes. The sciatic nerve segment containing the hip ligature was then dissected out and placed in the same fixative for an additional hour, before being transferred to 30% sucrose in sodium phosphate buffer.

The still-intact nerve bundle was then embedded in OCT compound (Tissue-Tek, Sakura Finetechnical Company Ltd., Tokyo, Japan), and frozen. Longitudinal cryostat sections (50 microns thick) were cut from the embedded and frozen nerve bundles, using a microtome.

Selected longitudinal tissue sections that had been cut from near the center of the nerve bundle were then treated with immunoreagents, to reveal the presence and concentration of phage particles. One reagent was a rabbit-derived antibody preparation (Sigma Chemicals, catalog number B2661) that binds to the pIII capsid protein on bacteriophage particles, and that also contains a Biotin polypeptide sequence. These antibodies were incubated with the tissue slice for 1 hour at room temperature. Alexa Fluor 488 (Molecular Probes, catalog number S-11223), which contains a streptavidin sequence that binds very tightly to the Biotin sequence on the phage-binding rabbit antibody, was then added and incubated for 1 hour at room temperature.

Fluorescent photographs were taken of the nerve bundle, covering a portion of the nerve bundle which included segments of nerve fibers on both sides of the hip ligature. One of those photographs, reproduced in black and white, is provided as FIG. 2 in the drawings.

That photograph (and others which showed very similar results) clearly shows that antibody-phage conjugates did indeed accumulate on the distal side of the hip ligature. These and similar photographs from other tests provide clear confirmation that the binding, endocytosis, and transport mechanisms described herein are indeed working efficiently, and in the manner disclosed.

Similar photographs and other analytical tests of a control treatment, which involved injecting M13KO7 phages without any receptor-specific internalizing antibodies crosslinked to them, showed no control phages at the same location, on the distal side of the ligatures.

The foregoing tests and results, using monoclonal antibodies affixed to phage particles, confirmed several key aspects of the invention disclosed herein. Among other things, these results confirmed that: (i) complete filamentous phage particles that could bind to p75 receptors in rat neurons could be internalized and then retrogradely transported, by sciatic nerve fibers; and, (ii) the ligation, emplacement, and harvesting protocols described above can be used satisfactorily and effectively, to accomplish *in vivo* screening and selection of particular ligands that can enable complete, viable, and infective phage particles to be internalised into, and then transported within, neuronal fibers, based on phage-fiber

contacts that occur outside the blood-brain barrier.

Based on that confirmation of their general approach to *in vivo* screening using nerve fibers, and after conceiving, developing, optimizing, and confirming a combination of methods and reagents that enabled these types of *in vivo* screening tests to be carried out successfully and effectively, the Applicants then extended their approach, by testing it in actual *in vivo* screenings of phage display libraries containing huge numbers of candidate ligands.

EXAMPLE 28: IN VITRO BIOPANNING OF scFv PHAGE LIBRARY

As mentioned above, the p75 receptor in rat neurons is known to have endocytotic activity. It is also known to have its copy numbers, on neuronal surfaces, increased by a factor of roughly 10 to 15 fold, in response to various types of neuronal injuries. In rats, this type of injury can be created, in a controlled and reproducible manner, by emplacement of a tight ligature loop around the sciatic nerve bundle, in a location slightly above the site of the tibial branch bifurcation, where the sciatic nerve divides into two major branches. A ligature at that site will provoke a substantial increase in p75 receptor numbers along the length of the sciatic nerve fibers between the spinal cord and the ligature site, and the affected nerve fibers segments with increased p75 receptor numbers will be long enough to enable emplacement of a phage-containing gel at one location, followed by harvesting of internalized and transported phages at a separate location.

All of these factors made the p75 receptor a useful and controllable target for initial testing and confirmation of the *in vivo* selection process disclosed herein, using phage display libraries with huge numbers of candidate ligands.

Accordingly, the Applicants chose to limit their first tests of the scFv phage display library to candidate ligands that could bind specifically to the p75 receptor in particular. Although the Applicants realized that other ligands in the display library (which, as mentioned in Example 22, contains an estimated 13 billion different variable fragment antibody sequences, designed to emulate the immune systems of ten different humans from widely varying ancestries) would inevitably be able to bind to other neuronal surface receptors that could trigger endocytosis and retrograde transport, their decision and goal, in their tests using the scFv library, was to limit their tests to ligands that would bind to the p75 receptor.

After an initial set of efforts, which confirmed the general principles but which also

led to wide variations in the resulting data (those tests and results are described in Example 30, and arose when the Applicants began trying to evaluate cyclic repetition of the in vivo screening process, using phages selected in one cycle of tests as the starting population for the next cycle), the Applicants decided to focus on p75 receptor endocytosis by processing the scFv phage display library using an *in vitro* procedure called "biopanning".

This in vitro method used a preparation of recombinant human p75 receptor polypeptides. These polypeptides, which are commercially available, encode amino acid residues 1-250 of the extracellular domain of human nerve growth factor (NGF) receptors, fused to a carboxy terminal 6x histidine-tagged Fc region of human IgG1 protein, via a peptide linker. In order to obtain glycosylated proteins, the chimeric protein is expressed in eukaryotic rather than bacterial cells, using an insect cell line known as Sf21 (from the "fall armyworm" moth, *Spodoptera frugiperda*), and a baculovirus expression vector. The recombinant mature chimeric protein exists as a disulfide-linked homodimer. Each monomer contains 466 amino acids and has a calculated mass of 51 kDa; however, because glycosylation increases the size and weight of the protein, each monomer migrates as a 90-100 kDa protein, when processed by electrophoresis in sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels.

These polypeptides were coated onto the surfaces of immunotubes. This was done by using 7 mL MAXISORP™ tubes, with a polystyrene hydrophilic surface (catalog #444474, from Nalge Nunc International, Denmark). A concentration of 5μg/mL of the recombinant human p75 receptor protein, suspended in 0.5 ml phosphate-buffered saline (PBs), was incubated overnight in the immunotubes at 4°C.

The next day, the tubes were poured out, and then rinsed with PBS, filled to the brim with 3% w/v skim milk in PBS, and blocked for 2 hours at room temperature (RT). Meanwhile, 50 μl of the scFv phage display library was pre-blocked with 450 μl of 3% skim milk in PBS in an Eppendorf tube for 1 hour at RT. The immunotubes were rinsed with PBS, then 500 μl of pre-blocked phage solution was added to the tubes, and incubated for 2 hours at RT.

After 15 washes with PBS containing 0.1% Tween 20, the remaining bound phages were eluted with 15 minute incubation of 500 μL fresh triethylamine (TEA) in a 100 mM, pH 11 solution at RT. The eluted phage were transferred into an Eppendorf tube, and neutralized by adding 250 μL of 1M Tris-HCl buffer, pH 7.4.

Half of the elutant was used to infect TG1 *E. coli* host cells, which were cultured to

an OD_{600} optical density of 0.5-0.8. The other half of the elutant was stored as a backup. The biopanned phage preparation was incubated with TG1 cells for 1 hour at 37°C, with 30 minutes under stationary, then 30 minutes of 300 rpm shaking. The infected cell solutions were then serially diluted and plated on 2TY agar plates with ampicillin, to determine phage titer.

Remaining cells were plated on four 243 mm x 243 mm 2TY agar plates with ampicillin, for amplification. These plates were incubated at 30°C overnight. Colonies were scraped into liquid 2TY broth, and grown to OD_{600} levels of 0.5-0.8. M13KO7 helper phages were then added, to form a final concentration of 5×10^9 "colony forming units" (cfu) per mL. The mixture was incubated for 30 minutes while stationary, then for 30 minutes in a shaker tray at 200 rpm. The cells were then centrifuged at 3500 rpm for 10 minutes, and the cell pellet was resuspended in 25 mL prewarmed 2TY (without glucose) containing kanamycin (50 μg/mL) and ampicillin (100 μg/mL). These were incubated overnight at 25°C, with rapid shaking, to produce phage particles. The phage particles were precipitated, using 20% polyethylene glycol (PEG) and 2.5 M NaCl. These particles were then mixed with a collagen gel, and a bolus of gel containing roughly 50 billion (5×10^{10}) cfu of phages was emplaced in a rat leg during an *in vivo* screening operation.

Only a single round of *in vitro* biopanning was used prior to *in vivo* screening, because the Applicants wanted to preserve maximal diversity of the p75-binding ligands that would be tested *in vivo*. This diversity would have been jeopardized by successive *in vitro* screenings, because it is known that many types of p75-binding antibodies are not internalized by cells having p75 receptors. The concern is that tightly-binding ligands appear to somehow lock up and/or contort *in vivo* p75 receptors, in ways that impede the ability of the p75 receptors to carry out the normal process of endocytosis. Since additional rounds of *in vitro* biopanning would tend to select for tight-binding ligands without regard to their ability to trigger endocytosis, it could lead to elimination of candidate ligands that might be substantially more effective in achieving actual endocytotic transport into cell interiors, *in vivo*.

Nevertheless, three successive rounds of biopanning were carried out, using the scFv phage library, to evaluate how this library would respond to repeated rounds of *in vitro* screening. To establish comparable results, each solution of phages used in the second and third rounds was diluted, by PBS, to match the titer of the solution that had been used in the first round.

While the first round of biopanning resulted in scFv titers of roughly 3000 cfu (compared to control values of roughly 1200 cfu, when PBS was tested), the second round of biopanning resulted in major increases, to about 84 million cfu. A third round of biopanning resulted in titers of about 85 million cfu, which was not a significant increase over the second round.

EXAMPLE 29: IN VIVO (SCIATIC NERVE) SELECTION OF INTERNALISED PHAGES FROM THE scFv LIBRARY

As mentioned in the previous example, the "enriched" portion of the scFv phage display library that was selected by one round of biopanning (using human p75 receptor polypeptides), as described in Example 29, was used as the starting reagent in a series of *in vivo* screenings in rats. These *in vivo* screenings used the procedures and methods that had been developed, tested, and optimized by using MC192/M12KO7 antibody-phage conjugates as described in Examples 25 and 26.

Briefly, in a first operation, an initial ligature was placed just above the tibial branching of the sciatic nerve, to induce increased p75 receptor expression on the sciatic nerve fibers above the ligature. A week later, in a second operation, the sciatic nerve bundle was cut, and the cut end was packed inside a silicone rubber sleeve with collagen gel containing about 50 billion cfu of scFv phages that had been obtained by a single round of p75 biopanning. During the second operation, a ligature was also emplaced and tightened around the sciatic nerve in the hip region, to create an obstacle that would cause internalised and retrogradely transported phage particles to accumulate, inside the nerve fibers, just distal to the ligature. Eighteen hours later, in a third operation, the rat was sacrificed and a segment of nerve fibers was harvested, including the hip ligature and roughly half a centimeter of nerve fibers distal to the ligature. The harvested nerve fibers were washed, cut into small pieces, and treated to remove and isolate phage particles. The phage particles were amplified, and titers were determined, using *E. coli* cells and helper phages.

While the absolute number of phage recovered from an excised nerve segment varied between experiments, a standardized measure of uptake and transport was generated, by always testing a control phage population, and comparing the results to the data from the test phage population.

To illustrate, using absolute numbers that resulted from a representative experiment

(n=3 for both control and test selections), 5 x 10^{10} (i.e., 50 billion) cfu (titered estimate) of control phages (unmodified M13KO7 helper phages) were emplaced into the phage contact site. The number of control phages that were recovered from the nerve segment excised 18 hours later was titered, giving a value of 14,650 (± 2,975, standard error of the mean (SEM), n=3). The same quantity (5 x 10^{10} cfu) of scFv-phage (biopanned once to recognize p75, as described in Example 28) was emplaced in a phage contact site, and the number of scFv phages recovered from the nerve segment excised 18 hours later was titered at 190400 (± 14,415 SEM, n=3). By comparing those two results, it was calculated that 13-fold more scFv phage (biopanned once for p75) were recovered from the excised nerve segments, than control phage. This experiment was repeated 3 times, with similar results each time.

To test whether this marked increase in uptake and transport of scFv phage was indeed the result of the scFv binding to p75, the experiment was repeated in other sets of animals, in which the sciatic nerve had not been pre-ligated (and, therefore, the motor neurons had not upregulated their expression of p75 above the very low and frequently undetectable levels that appear in rats that are more than about 2 weeks old). In these tests, the amounts of control phage (M13KO7) and test phage (scFv) that were applied were held the same as before, at 5 x 10^{10} cfu. The number of control phage that were recovered from nerve segments excised 18 hours later was 14,815 ± 4,481 (n=3). The amount of scFv phage (biopanned once for p75 recognition) that were recovered from nerve segments excised 18 hours later was 16,413 ± 4,541 (n=3). These data clearly showed that the efficiency of cellular intake and transport of scFv phage (biopanned once for p75 recognition) was essentially no different from that of control phage, when rats were tested that had very low levels of p75 receptors, as occurs naturally in rats that are six weeks of age or older. This confirmed that there was a clear relationship between p75 expression levels, and efficiency of uptake and transport of scFv phage that had been biopanned to recognize p75 receptors. This experiment was repeated 3 times, with similar results.

EXAMPLE 30: CYCLIC TESTING OF UNPANNED scFv LIBRARY

In a series of tests that were performed before the *in vitro* biopanning procedure (described in Example 28) was settled upon and used, the scFv library was tested in a series of cyclic *in vivo* screening tests, using sciatic nerve fibers as described in Examples 25 and 26. These tests are referred to as "cyclic", because a screened and selected phage

population that was obtained from one round of tests was then used as a starting reagent, in the next cycle of tests.

Although these tests provided clear evidence that the phage libraries contained particular phage-borne ligands that activated and drove endocytotic internalisation and retrograde transport, the results of these cyclic tests were highly variable. The data scattering led the Applicants to conclude that the data were consistent with the following interpretations:

(i) the ligand-receptor binding and uptake process was saturable, due to the limited number of p75 receptors on the surfaces of the nerve fibers;

(ii) the input phage populations were highly diverse, with only one or a few copies of any one particular phage present in any initial round(s), and with subsequent rounds likely to contain hundreds or even thousands of different phage candidates;

(iii) therefore, the probability was quite low that any one particular phage would be selected in two different experiments on different animals (this probability can be regarded as being roughly equal to the number of copies of any one particular phage in a test population, divided by the number of alternative phages that the nerve bundle could effectively sample from).

These factors clearly can account for the very high variability seen between different experiments using different animals. Therefore, after encountering and pondering those high levels of variability, the Applicants decided to experiment with a pre-screening step (i.e., *in vitro* biopanning) that would reduce the variation within the input population, and that would also substantially increase the number of multiple copies of p75-binding phage candidates that would be available for the nerve bundle to sample from.

The data obtained from the scFv library tests that were done prior to the pre-screening step did indicate that the first, the second, and possibly the third successive screening cycles all appeared to lead to greater efficiencies, in internalisation and retrograde transport by the cells. However, under the particular conditions that were used, those efficiencies tended to drop off if still more cycles of *in vivo* screening were used. While the cause for the eventual fall-off was not clear, a significant proportion of individual colonies of phage selected from the second or third round selections can reasonably be anticipated to display ligands that bind to neuronal receptors and stimulate internalisation and retrograde transport (since they were repeatedly selected, the probability that they might be false positives is low).

Cyclic *in vivo* screening has not yet been evaluated thoroughly, and in particular, it has not yet been tested with a phage population that has been pre-screened, by biopanning or similar efforts. Nevertheless, the work done to date is believed to clearly demonstrate and confirm that:

(1) with at least some types of phage populations, a series of cyclic *in vivo* screenings, where a candidate population that has been selected by one round of screening is used as a starting reagent in the next round of screening, is indeed possible, and in at least some cases is likely to help identify and isolate candidate ligands that are exceptionally effective in triggering and driving endocytotic transport into cells; and,

(2) it is feasible to develop numerical indices that will provide useful indicators of when a cyclic process should be stopped, to allow careful analysis and sequencing of candidate ligands that appear to offer the best performers that have been identified up until that point in the screening process.

(3) it is also likely that at least some of the phage ligands stimulated internalisation and retrograde transport after binding to surface molecules that were not previously known to mediate endocytotic events.

Finally, in considering the implications of these analyses, it should be borne in mind that the fundamental and overriding goal of this type of in vivo screening is not to create a highly enriched or "elite" phage population, that can offer many thousands of phage candidates that will be internalised by nerve fibers. Instead, the goal is to identify and isolate (and, in the case of polypeptide ligands, to determine the nucleotide gene sequence and/or the amino acid polypeptide sequence of) just one or a small number of particular ligands that are highly effective in activating and drive the process of endocytotic internalisation. These are the types of ligands that, once they have been identified and isolated, can be replicated in mass, and incorporated into molecular complexes that will transport useful passenger or payload molecules into specific classes of cells that have specific targeted endocytotic receptors or similar molecules on their surfaces.

It must also be kept in mind that a biopanning step, as described above for using p75 polypeptide sequences to pre-screen the scFv phage library, can be carried out by using (as the "antigen" molecule that will be affixed to the surfaces of the immunotubes) any known polypeptide sequence or fragment, from any type of known or suspected endocytotic receptor, or from other surface molecule suspected of having endocytotic activity. It can also be carried out by using any glycosylated cell surface molecules that are suspected of

having endocytotic activity.

EXAMPLE 31: IN VIVO SELECTION, USING PhD-C7C PHAGE LIBRARY

As briefly mentioned in Example 22, the Ph.D-C7C phage display library contains an estimated two billion different phages, with foreign DNA inserts that encoding random sequences of seven amino acids, inserted near the DNA sequence that encodes the N-terminus of the pIII capsid protein of M13 phages. This library provided an essentially random repertoire of peptide sequences that could be tested, to determine whether certain phages would be internalized and transported by neurons in the sciatic nerve bundle.

In vivo screening of the PhD-C7C library, using the sciatic nerve procedures disclosed above, indicated that this library performed just as expected. Substantial numbers of phages were internalized by the sciatic nerve fibers, and transported to a phage accumulation zone immediately distal to the hip ligature. Selected phages were removed, in viable form, from the harvested nerve segments, and those p75-selected viable phages could be replicated and manipulated in any way of the ways described above.

If desired, as indicated above, the PhD-C7C library also can be pre-screened, using a biopanning technique (as described above for the p75 biopanning of the scFv library), using any known and available type of receptor polypeptide sequence as the biopanning antigen.

Thus, there has been shown and described a new and useful method for (i) using *in vivo* screening, to identify ligands that can efficiently activate and drive the process of cellular endocytosis, via selected endocytotic molecules that are present on the surfaces of only limited numbers and types of cells, and (ii) incorporating those ligands into molecular complexes that can be used to efficiently transport useful passenger or payload molecules into cells having the targeted endocytotic receptors or other surface molecules. Although this invention has been exemplified for purposes of illustration and description by reference to certain specific embodiments, it will be apparent to those skilled in the art that various modifications, alterations, and equivalents of the illustrated examples are possible. Any such changes which derive directly from the teachings herein, and which do not depart from the spirit and scope of the invention, are deemed to be covered by this invention.

Thus, there has been shown and described a new and useful means for non-invasive transport of therapeutic or other useful polypeptides through the BBB, into brain and spinal tissue (and in particular to neurons that lie wholly within the BBB), and for using *in vivo*

screening to identify ligands that can efficiently activate and drive the process of cellular endocytosis, via selected endocytotic molecules that are present on the surfaces of only limited numbers and types of cells. Although this invention has been exemplified for purposes of illustration and description by reference to certain specific embodiments, it will be apparent to those skilled in the art that various modifications, alterations, and equivalents of the illustrated examples are possible. Any such changes which derive directly from the teachings herein, and which do not depart from the spirit and scope of the invention, are deemed to be covered by this invention.

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CLAIMS

1. A method for delivering neuroactive polypeptide molecules through a blood-brain barrier in a higher animal, comprising the steps of:

a. creating a genetic vector which carries at least one gene which encodes a neuroactive polypeptide, and which is constructed in a manner which will enable the genetic vector to transfect at least one class of accessible neurons which straddle the blood-brain barrier, each such accessible neuron having at least one peripheral projection that is accessible to compounds that have not penetrated the animal's blood-brain barrier, if said genetic vector is administered to such higher animal by means which cause direct contact between at least one copy of the genetic vector and at least one such peripheral projection, wherein the gene which encodes the neuroactive polypeptide is capable of expressing copies of the neuroactive polypeptide within such accessible neurons after such accessible neurons have been transfected by said genetic vector; and,

b. administering the genetic vector to the higher animal in a location and manner which ensure that: (i) at least one copy of the genetic vector contacts and transfects at least one such accessible neuron; and (ii) at least one such accessible neuron expresses the neuroactive polypeptide encoded by the gene and subsequently secretes the neuroactive polypeptide into central nervous system tissue which is protected by the blood-brain barrier.

2. The method of Claim 1, wherein the accessible neurons which straddle the blood-brain barrier are sensory neurons.

3. The method of Claim 2, wherein the sensory neurons are olfactory receptor neurons.

4. The method of Claim 2, wherein the sensory neurons are nocioceptive neurons.

5. The method of Claim 1, wherein the accessible neurons which straddle the blood-brain barrier are motor neurons.

6. The method of Claim 5, wherein the motor neurons are spinal motor neurons.

7. The method of Claim 5, wherein the motor neurons are motor neurons of the hypoglossal nucleus.

8. The method of Claim 1, wherein the accessible neurons which straddle the blood-brain barrier are pre-ganglionic neurons of the parasympathetic nervous system.

9. The method of Claim 1, wherein the accessible neurons which straddle the blood-brain barrier are pre-ganglionic neurons of the sympathetic nervous system.

10. The method of claim 1, wherein the neuroactive polypeptide is selected from the group consisting of neurotrophic factors, endocrine factors, growth factors, paracrine factors, hypothalamic releasing factors, neurotransmitter polypeptides, antibodies and antibody fragments which bind to neurotrophic factors, antibodies and antibody fragments which bind to neurotrophic factor receptors, polypeptide antagonists, agonists for a receptor expressed by a CNS cell, and polypeptides involved in lysosomal storage disease.

11. The method of claim 1, wherein the genetic vector contains cell transfection components that include at least a portion of a viral capsid active on a mammalian cell.

12. The method of claim 1, wherein the genetic vector comprises a cationic liposome or other gene transfection lipid.

13. The method of claim 1, wherein the genetic vector comprises a ligand which binds in a specific manner to at least one type of endocytotic receptor on the accessible neurons.

14. The method of claim 13, wherein the ligand has been identified through selection steps which include:

(a) subjecting a multiplicity of neurons of a selected type to contact by a phagemid or phage display library, each phage in the library carrying a candidate DNA sequence and having on an exposed phage surface a polypeptide encoded by the candidate DNA sequence, and the phage display library consisting of multiple phage particles with multiple candidate DNA sequences and corresponding polypeptides exposed on the phage surfaces;

(b) selecting at least one clonal phage line from the phage display library, wherein the selected clonal phage line was transported into neurons of the selected type.

15. The method of claim 1, wherein the genetic vector comprises a macromolecule that has been shown to promote transneuronal passage of associated genetic material out of a selected type of accessible neuron which straddles the blood-brain barrier and into at least one type of neuron which is positioned entirely within central nervous system tissue and protected by the blood-brain barrier of an animal.

16. The method of claim 1, wherein the genetic vector encodes a chimeric polypeptide comprising:

a. a leader sequence which enables or promotes anterograde transport of the chimeric polypeptide within a neuron that expresses the chimeric polypeptide; and,

b. a neuroactive sequence which can create a therapeutic benefit by reacting with cells located wholly within CNS tissue that is protected by the blood-brain barrier, after secretion within the blood-brain barrier by an accessible neuron which straddles the blood-brain barrier.

17. The method of claim 1, wherein the genetic vector encodes a chimeric polypeptide comprising:

a. a leader sequence which enables or promotes secretion of the chimeric polypeptide at neuronal termini located within CNS tissue that is protected by the blood-brain barrier of an animal; and,

b. a neuroactive sequence which can create a therapeutic benefit by reacting with cells located wholly within CNS tissue that is protected by the blood-brain barrier, after secretion within the blood-brain barrier by an accessible neuron which straddles the blood-brain barrier.

18. A method for delivering neuroactive polypeptide molecules through a blood-brain barrier in a higher animal, comprising the step of administering to the higher animal a genetic vector which carries at least one gene which encodes a neuroactive polypeptide,

wherein said genetic vector has been constructed in a manner which enables the genetic vector to transfect at least one class of accessible neuron which straddles the blood-

brain barrier, if administered to such higher animal in a location and manner which causes direct contact between the genetic vector and such accessible neurons which straddle the blood-brain barrier,

and wherein said genetic vector is administered to such higher animal in a location and manner which ensures that: (i) at least one copy of the genetic vector contacts and transfects at least one such accessible neuron; (ii) at least one transfected accessible neuron expresses copies of at least one neuroactive polypeptide encoded by at least one gene carried by said genetic vector; and, (iii) at least one transfected accessible neuron secretes copies of at least one such neuroactive polypeptide, at a location within central nervous system tissue protected by the blood-brain barrier.

19. The method of Claim 18, wherein the genetic vector has been designed to transfect sensory neurons.

20. The method of Claim 18, wherein the genetic vector has been designed to transfect motor neurons.

21. The method of Claim 18, wherein the genetic vector has been designed to transfect pre-ganglionic neurons of the parasympathetic nervous system.

22. The method of Claim 18, wherein the genetic vector has been designed to transfect pre-ganglionic neurons of the sympathetic nervous system.

23. The method of claim 18, wherein the neuroactive polypeptide is selected from the group consisting of neurotrophic factors, endocrine factors, growth factors, paracrine factors, hypothalamic release factors, neurotransmitter polypeptides, antibodies and antibody fragments which bind to neurotrophic factors, antibodies and antibody fragments which bind to neurotrophic factor receptors, polypeptide antagonists, agonists for a receptor expressed by a CNS cell, and polypeptides involved in lysosomal storage disease.

24. The method of claim 18, wherein the genetic vector contains cell transfection components that include at least a portion of a viral capsid active on a mammalian cell.

25. The method of claim 18, wherein the genetic vector comprises a cationic liposome or other gene transfection lipid.

26. The method of claim 18, wherein the genetic vector comprises a ligand which binds in a specific manner to at least one type of endocytotic receptor on the accessible neurons.

27. The method of claim 18, wherein the genetic vector comprises a macromolecule that has been shown to promote transneuronal passage of associated genetic material out of a selected type of accessible neuron which straddles the blood-brain barrier and into at least one type of neuron which is positioned entirely within central nervous system tissue and protected by the blood-brain barrier of an animal.

28. The method of claim 18, wherein the genetic vector encodes a chimeric polypeptide comprising:

a. a leader sequence which enables or promotes anterograde transport of the chimeric polypeptide within a neuron that expresses the chimeric polypeptide; and,

b. a neuroactive sequence which can create a therapeutic benefit by reacting with cells located wholly within CNS tissue that is protected by the blood-brain barrier, after secretion within the blood-brain barrier by an accessible neuron which straddles the blood-brain barrier.

29. The method of claim 18, wherein the genetic vector encodes a chimeric polypeptide comprising:

a. a leader sequence which enables or promotes secretion of the chimeric polypeptide at neuronal termini located within CNS tissue that is protected by the blood-brain barrier of an animal; and,

b. a neuroactive sequence which can create a therapeutic benefit by reacting with cells located wholly within CNS tissue that is protected by the blood-brain barrier, after secretion within the blood-brain barrier by an accessible neuron which straddles the blood-brain barrier.

30. A genetic vector designed to initiate and capable of initiating a neuronal process

that will non-invasively deliver neuroactive polypeptide molecules into central nervous system tissue protected by a blood-brain barrier in a higher animal, said genetic vector comprising cell transfection components and associated genetic material, which have been assembled into a genetic vector which is suited for and capable of transfecting at least one type of accessible neuron which straddles the blood-brain barrier and has at least one peripheral projection that is accessible to genetic vectors that have not penetrated an animal's blood-brain barrier,

wherein:

a. the cell transfection components are selected to cause the genetic vector to (i) bind to the selected type of accessible neuron, and (ii) insert associated genetic material carried by the genetic vector into the accessible neurons; and,

b. the associated genetic material contains at least one gene which encodes a neuroactive polypeptide, wherein said gene has a gene promoter that causes the gene to be expressed, inside transfected accessible neurons, into said neuroactive polypeptide or a precursor thereof, and wherein copies of the neuroactive polypeptide or precursor thereof have been shown to be (i) transported, within transfected accessible neurons, to neuronal secretion locations in central nervous system tissue that is protected by the blood-brain barrier of the animal, and (ii) secreted by transfected accessible neurons at such neuronal secretion locations, in central nervous system tissue that is protected by the blood-brain barrier of the animal.

31. The genetic vector of Claim 30, which has been proven in *in vivo* mammalian tests to be fully capable of initiating a neuronal process that will non-invasively deliver neuroactive polypeptide molecules, encoded by the genetic vector and expressed within transfected accessible neurons, into central nervous system tissue protected by a blood-brain barrier in at least one mammalian species.

32. The genetic vector of Claim 30, which has been proven in human clinical trials to be fully capable of initiating a neuronal process that will non-invasively deliver neuroactive polypeptide molecules, encoded by the genetic vector and expressed within transfected accessible neurons, into central nervous system tissue protected by a blood-brain barrier in treated human patients.

33. The genetic vector of Claim 30, which is designed to transfect at least one type of sensory neuron.

34. The genetic vector of Claim 33, which is designed to transfect olfactory receptor neurons.

35. The genetic vector of Claim 33, which is designed to transfect nocioceptive neurons.

36. The genetic vector of Claim 30, which is designed to transfect at least one type of motor neuron.

37. The genetic vector of Claim 30, which is designed to transfect at least one type of pre-ganglionic neuron belonging to a sympathetic or parasympathetic nervous system of a higher animal.

38. The genetic vector of claim 30, wherein the gene encodes a neuroactive polypeptide selected from the group consisting of neurotrophic factors, endocrine factors, growth factors, paracrine factors, hypothalamic release factors, neurotransmitter polypeptides, antibodies and antibody fragments which bind to neurotrophic factors, antibodies and antibody fragments which bind to neurotrophic factor receptors, polypeptide antagonists, agonists for a receptor expressed by a CNS cell, and polypeptides involved in lysosomal storage disease.

39. The genetic vector of claim 30, wherein the cell transfection components comprise at least a portion of a mammalian viral capsid.

40. The genetic vector of claim 30, wherein the cell transfection components comprise a cationic liposome.

41. The genetic vector of claim 30, wherein the cell transfection components comprise a ligand which binds in a specific manner to at least one type of endocytotic receptor on the accessible neurons.

42. The genetic vector of claim 41, wherein the ligand has been identified through selection steps which include:

(a) subjecting a multiplicity of neurons of a selected type to contact by a phagemid or phage display library, each phage in the library carrying a candidate DNA sequence and having on an exposed phage surface a polypeptide encoded by the candidate DNA sequence, and the phage display library consisting of multiple phage particles with multiple candidate DNA sequences and corresponding polypeptides exposed on the phage surfaces;

(b) selecting at least one clonal phage line from the phage display library, wherein the selected clonal phage line was efficiently transported into neurons of the selected type.

43. The genetic vector of claim 30, wherein the cell transfection components comprise a polypeptide that has been shown to promote transneuronal passage of associated genetic material out of a selected type of accessible neuron which straddles the blood-brain barrier and into at least one type of neuron which is positioned entirely within central nervous system tissue and protected by the blood-brain barrier of an animal.

44. The genetic vector of claim 30, which encodes a chimeric polypeptide comprising:

a. a leader sequence which enables or promotes anterograde transport of the chimeric polypeptide within a neuron that expresses the chimeric polypeptide; and,

b. a neuroactive sequence which can create a therapeutic benefit by reacting with cells located wholly within CNS tissue that is protected by the blood-brain barrier, after secretion within the blood-brain barrier by an accessible neuron which straddles the blood-brain barrier.

45. The genetic vector of claim 30, which encodes a chimeric polypeptide comprising:

a. a leader sequence which enables or promotes secretion of the chimeric polypeptide at neuronal termini located within CNS tissue that is protected by the blood-brain barrier of an animal; and,

b. a neuroactive sequence which can create a therapeutic benefit by reacting with cells located wholly within CNS tissue that is protected by the blood-brain barrier, after secretion within the blood-brain barrier by an accessible neuron which straddles the blood-

brain barrier.

46. A genetic vector designed to initiate and capable of initiating a neuronal process that will non-invasively deliver neuroactive polypeptide molecules into central nervous system tissue protected by a blood-brain barrier in a higher animal, wherein the genetic vector encodes a chimeric polypeptide comprising:

a. a leader sequence which enables or promotes at least one activity elected from the group consisting of:

(i) anterograde transport of the chimeric polypeptide within a neuron that expresses the chimeric polypeptide; and,

(ii) secretion of the chimeric polypeptide at neuronal termini located within CNS tissue that is protected by the blood-brain barrier of an animal; and,

b. a neuroactive sequence which can create a therapeutic benefit by reacting with cells located wholly within CNS tissue that is protected by the blood-brain barrier, after secretion within the blood-brain barrier by a neuron which straddles the blood-brain barrier.

47. The genetic vector of Claim 46, wherein the neuroactive sequence of the polypeptide is selected from the group consisting of neurotrophic factors, endocrine factors, growth factors, paracrine factors, hypothalamic release factors, neurotransmitter polypeptides, antibodies and antibody fragments which bind to neurotrophic factors, antibodies and antibody fragments which bind to neurotrophic factor receptors, polypeptide antagonists, agonists for a receptor expressed by a CNS cell, and polypeptides involved in lysosomal storage disease.

48. A molecular complex, comprising:

a. at least one ligand component that was identified by a process of in vivo selection that required endocytotic uptake into neurons for such selection to occur, and,

b. at least one passenger component capable of causing a desired effect after such passenger component has been transported into at lease one type of targeted animal cell having endocytotic surface molecules to which the ligand component which will specifically bind.

49. The molecular complex of Claim 48, wherein the molecular complex can enable

ligand-mediated ligand-mediated endocytotic transport of the passenger component into at least one class of targeted animal cells having endocytotic surface molecules to which the ligand will specifically bind, if the molecular complex is administered into an animal having said targeted animal cells.

50. The molecular complex of Claim 48, wherein the ligand component was derived from a molecule or molecular complex harvested from a neuron in vivo.

51. The molecular complex of Claim 48, which also comprises at least one coupling component that couples the passenger component to the ligand component in a manner that enables the passenger component to enter at least one class of targeted mammalian cells when the molecular complex undergoes ligand-mediated endocytotic transport into such cells.

52. The molecular complex of Claim 48, wherein the process of *in vivo* selection of the ligand component also required intracellular transport within neuronal fibers, following endocytotic uptake of the ligand component into the neurons.

53. The molecular complex of Claim 48, wherein the process of *in vivo* selection comprised the following steps:

(1) emplacing a multiplicity of candidate ligand components into an emplacement site in a living animal, in a manner that caused contact between said candidate ligand components, and surfaces of neurons; and,

(2) harvesting, at a harvesting site located away from the emplacement site, segments of neuronal fibers that contained ligand components that were internalised by the neurons and transported by the neuronal fibers.

54. The molecular complex of Claim 53, wherein the candidate ligand components were exposed on phage particle surfaces in a phage display library, during the *in vivo* selection process.

55. The molecular complex of Claim 53, wherein the candidate ligand components were generated by a process of combinatorial chemical synthesis.

56. The molecular complex of Claim 53, wherein the candidate ligand components were processed by an affinity binding step prior to the *in vivo* selection process.

57. The molecular complex of Claim 48, wherein the ligand component specifically binds to low-affinity nerve growth factor receptors.

58. A molecular complex, comprising:

a. at least one endocytotic ligand component that was selected by a process of *in vivo* selection that required endocytotic uptake through a targeted class of endocytotic surface molecules for such *in vivo* selection to occur; and,

b. at least one passenger component capable of causing a desired effect after such passenger component has been transported into at least one type of mammalian cell having the targeted class of endocytotic surface molecules.

59. The molecular complex of Claim 58, which also comprises at least one coupling component that couples the passenger component to the ligand component in a manner that enables the passenger component to enter at least one class of targeted mammalian cells when the molecular complex undergoes ligand-mediated endocytotic transport into such cells.

60. The molecular complex of Claim 58, wherein the process of *in vivo* selection of the ligand component required endocytotic entry of the ligand component into neuronal fibers.

61. The molecular complex of Claim 58, wherein the process of *in vivo* selection comprised the following steps:

(1) emplacement of a multiplicity of candidate ligand components into an emplacement site in a living animal, in a manner that caused contact between said candidate ligand components, and surfaces of neurons; and,

(2) harvesting, at a harvesting site located away from the emplacement site, segments of neuronal fibers that contained ligand components that were internalised by the neurons and transported by the neuronal fibers.

62. The molecular complex of Claim 61, wherein the candidate ligand components were exposed on phage particle surfaces in a phage display library, during the *in vivo* selection process.

63. The molecular complex of Claim 61, wherein the candidate ligand components were generated by a process of combinatorial chemical synthesis.

64. The molecular complex of Claim 61, wherein the candidate ligand components were processed by an affinity binding step prior to the *in vivo* selection process.

65. The molecular complex of Claim 61, wherein the ligand component specifically binds to low-affinity nerve growth factor receptors.

66. A purified preparation of endocytotic ligands, comprising a multiplicity of endocytotic ligands having a molecular structure that was identified by a process which included *in vivo* selection requiring endocytotic uptake into neurons for such *in vivo* selection to occur, and wherein said ligands are suited for being coupled to passenger components in a manner that will generate molecular complexes that can transport said passenger components into at least one class of targeted cells having endocytotic surface molecules to which the endocytotic ligands will specifically bind.

67. The purified preparation of Claim 66, wherein the endocytotic ligands are substantially free of additional ligand candidates that cannot bind to endocytotic surface molecules to which the endocytotic ligands will specifically bind.

68. The purified preparation of Claim 66, wherein the process of *in vivo* selection also required intracellular transport within neuronal fibers, following endocytotic uptake of candidate ligand components into the neuronal fibers.

69. The purified preparation of Claim 66, wherein the process of *in vivo* selection comprised the following steps:

(1) emplacement of a multiplicity of candidate ligand components into an emplacement site in a living animal in a manner that caused contact between said candidate

ligand components, and surfaces of neurons; and,

(2) harvesting, at a harvesting site located away from the emplacement site, segments of neuronal fibers that contained ligand components that were internalised by the neurons and transported by the neuronal fibers.

70. The purified preparation of Claim 66, wherein candidate ligand components were exposed on phage particle surfaces in a phage display library, during the *in vivo* selection process.

71. The purified preparation of Claim 66, wherein candidate ligand components that were screened by the *in vivo* selection process were generated by a process of combinatorial chemical synthesis.

72. The purified preparation of Claim 66, wherein candidate ligand components that were screened by the *in vivo* selection process were processed by an affinity binding step prior to the *in vivo* selection process.

73. The purified preparation of Claim 66, wherein the ligand component specifically binds to low-affinity nerve growth factor receptors.

74. An *in vivo* selection process for isolating endocytotic ligands that can enable endocytotic uptake of molecular complexes, containing said endocytotic ligands coupled to passenger components, into targeted cells having endocytotic surface molecules to which the endocytotic ligands will specifically bind, comprising the following steps:

(1) emplacing a multiplicity of candidate ligand components into an emplacement site in a living animal, in a manner that caused *in vivo* contact between said candidate ligand components, and surfaces of neurons; and,

(2) harvesting, at a harvesting site located away from the emplacement site, segments of neuronal fibers that contained ligand components that were internalised by the neurons and transported by the neuronal fibers.

75. The *in vivo* selection process of Claim 74, wherein the candidate ligand components were exposed on phage particle surfaces in a phage display library, during the

in vivo selection process.

76. The *in vivo* selection process of Claim 74, wherein the candidate ligand components were generated by a process of combinatorial chemical synthesis.

77. The *in vivo* selection process of Claim 74, wherein the candidate ligand components were processed by an affinity binding step prior to the *in vivo* selection process.

78. The *in vivo* selection process of Claim 74, wherein the neuronal fibers are treated in a manner that will increase expression of low-affinity nerve growth factor receptors, prior to the *in vivo* selection process.

79. A method for introducing foreign molecules into a selected class of targeted animal cells, comprising the step of contacting the targeted animal cells with at least one copy of an endocytotic molecular complex that comprises:

a. at least one ligand component that was identified by a process of *in vivo* selection that required endocytotic uptake into neurons for such selection to occur, and,

b. at least one passenger component capable of causing a desired effect after such passenger component has been transported into a targeted mammalian cell having endocytotic surface molecules to which the ligand component will specifically bind.

80. The method of Claim 79, wherein the endocytotic molecular complex also comprises at least one coupling component that couples the passenger component to the ligand component in a manner that enables the passenger component to enter the targeted mammalian cells when the molecular complex undergoes ligand-mediated endocytotic transport into such cells.

81. The method of Claim 79, wherein the process of *in vivo* selection comprised the following steps:

(1) emplacing a multiplicity of candidate ligand components into an emplacement site in a living animal, in a manner that caused contact between said candidate ligand components, and surfaces of neurons; and,

(2) harvesting, at a harvesting site located away from the emplacement site, segments of neuronal fibers that contained ligand components that were internalised by the neurons and transported by the neuronal fibers.

82. The method of Claim 31, wherein the candidate ligand components were exposed on phage particle surfaces in a phage display library, during the *in vivo* selection process.

83. The method of Claim 32, wherein the candidate ligand components were generated by a process of combinatorial chemical synthesis.

84. The method of Claim 32, wherein the candidate ligand components were processed by an affinity binding step prior to the *in vivo* selection process.

85. The method of Claim 79, wherein the neuronal fibers were treated in a manner that increased expression of low-affinity nerve growth factor receptors, prior to the *in vivo* selection process.

86. The method of Claim 79, wherein the ligand component specifically binds to low-affinity nerve growth factor receptors.

87. A phage display library, comprising a multiplicity of phages that display at least one candidate ligand sequence in at least one coat protein, wherein said phage display library has been prescreened, by an affinity binding step which utilized affinity binding to a polypeptide that is known to have endocytotic activity on animal cells, to select phage particles that are included in the library.

88. A purified genetic vector which encodes a polypeptide sequence, wherein said polypeptide sequence comprises an endocytotic ligand sequence that was identified by a process of *in vivo* selection that required endocytotic uptake into neurons for such selection to occur.

89. The purified genetic vector of Claim 88, wherein the process of *in vivo* selection

comprised the following steps:

(1) emplacing a multiplicity of candidate ligand components into an emplacement site in a living animal, in a manner that caused contact between said candidate ligand components, and surfaces of neurons; and,

(2) harvesting, at a harvesting site located away from the emplacement site, segments of neuronal fibers that contained ligand components that were internalised by the neurons and transported by the neuronal fibers.

90. The purified genetic vector of Claim 88, wherein the candidate ligand components were exposed on phage particle surfaces in a phage display library, during the *in vivo* selection process.

91. The purified genetic vector of Claim 88, wherein the candidate ligand components were generated by a process of combinatorial chemical synthesis.

92. The purified genetic vector of Claim 88, wherein the candidate ligand components were processed by an affinity binding step prior to the *in vivo* selection process.

93. The purified genetic vector of Claim 88, wherein the ligand component specifically binds to low-affinity nerve growth factor receptors.

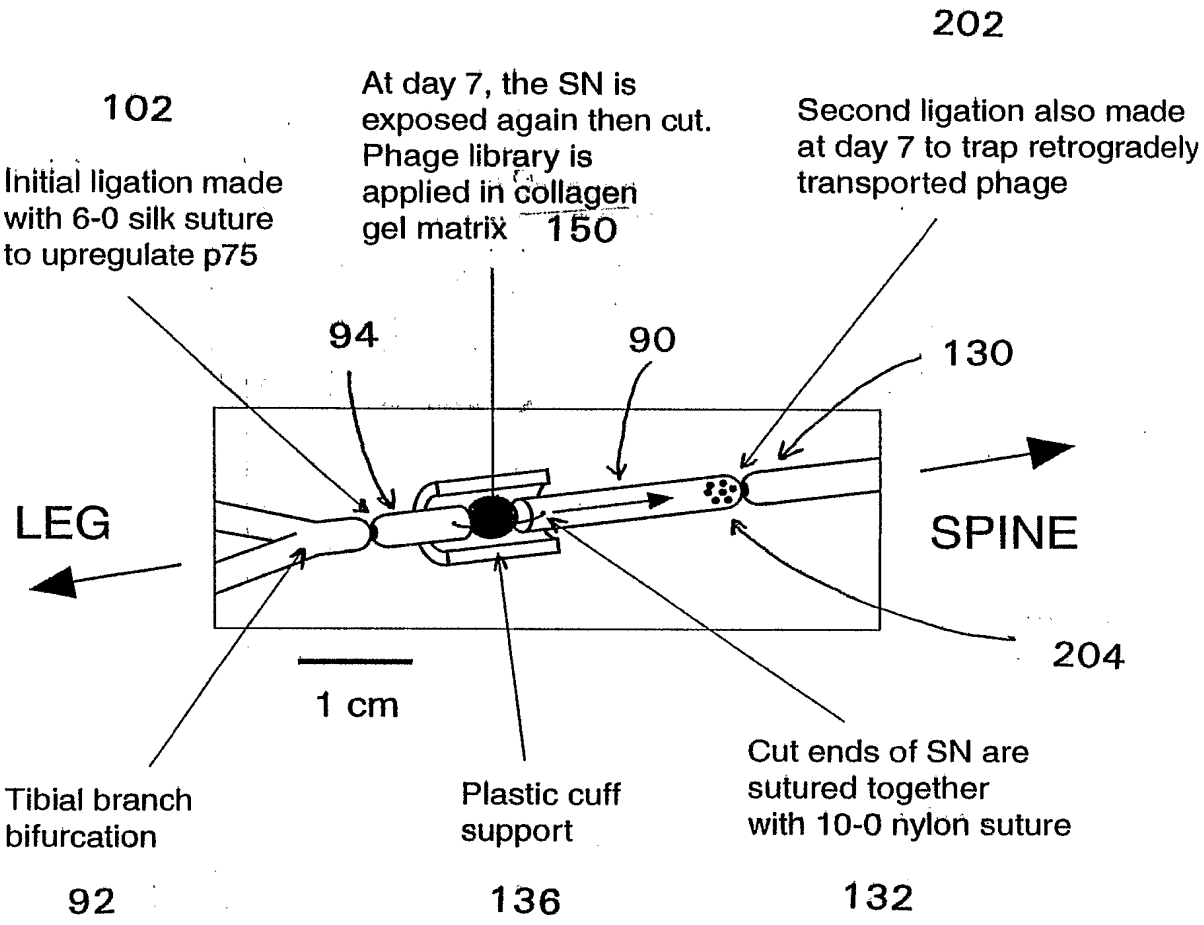
202
102
Initial ligation made with 6-0 silk suture to upregulate p75
At day 7, the SN is exposed again then cut. Phage library is applied in collagen gel matrix
150
Second ligation also made at day 7 to trap retrogradely transported phage
94
90
130
LEG
SPINE
204
1 cm
Tibial branch bifurcation
92
Plastic cuff support
136
Cut ends of SN are sutured together with 10-0 nylon suture
132

Fig. 1

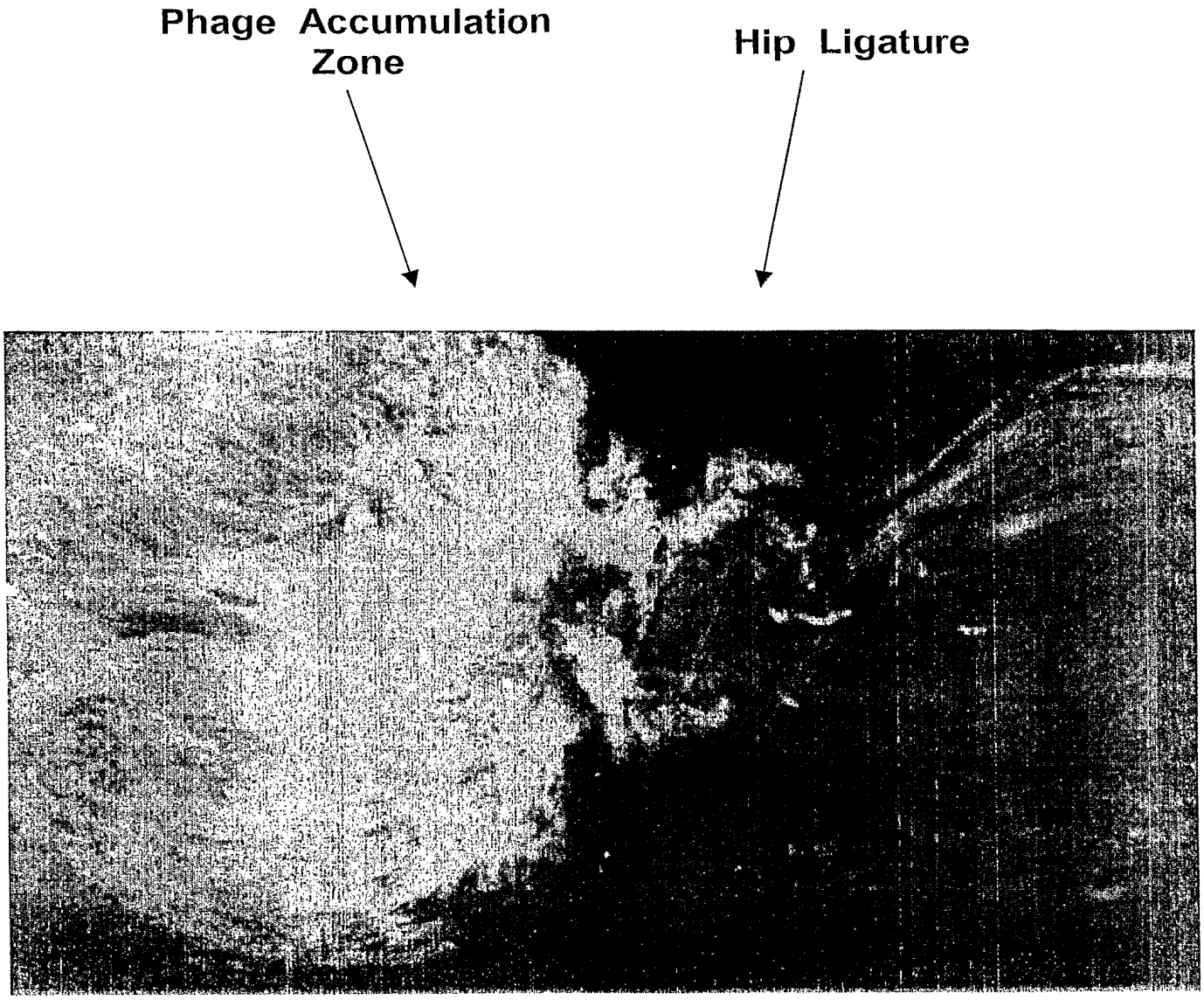

Fig. 2B

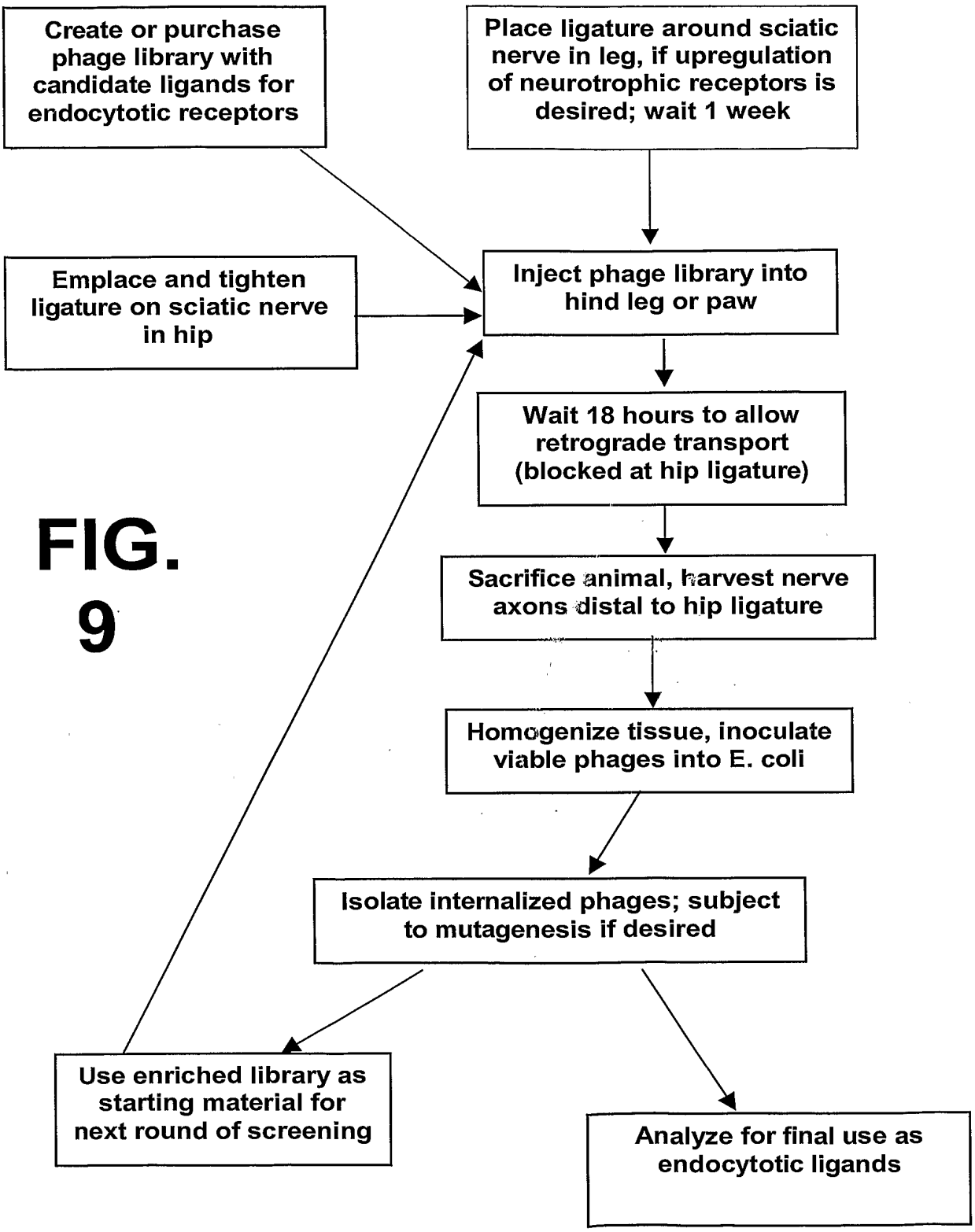
Create or purchase phage library with candidate ligands for endocytotic receptors
Place ligature around sciatic nerve in leg, if upregulation of neurotrophic receptors is desired; wait 1 week
Emplace and tighten ligature on sciatic nerve in hip
Inject phage library into hind leg or paw
Wait 18 hours to allow retrograde transport (blocked at hip ligature)
Sacrifice animal, harvest nerve axons distal to hip ligature
Homogenize tissue, inoculate viable phages into E. coli
Isolate internalized phages; subject to mutagenesis if desired
Use enriched library as starting material for next round of screening
Analyze for final use as endocytotic ligands

FIG. 9